Toxicological profile for

Carrageenan

This ingredient has been assessed to determine potential human health effects for the consumer. It was considered not to increase the inherent toxicity of the product and thus is acceptable under conditions of intended use.

1. Name of substance and physico-chemical properties

1.1. IUPAC systematic name

Not applicable.

1.2. Synonyms

3,6-Anhydro-D-galactan; Aubygel GS; Aubygum DM; Burtonite V-40-E; Carastay; Carastay C; Carrageen; Carrageenan; Carrageenan gum; Carrageenan [EP]; Carrageenan, degraded; Carrageenan, native; Carrageenin; Carragheanin; Carragheen; Carragheen gum; Carragheenan; Carragheenin; Carraguard; CCRIS 3956; Chondrus; Chondrus extract; Colloid 775; Coreine; EINECS 232-524-2; Eucheuma spinosum gum; FEMA No. 2596; Flanogen ELA; Galozone; Gelcarin; Gelcarin HWG; Gelozone; Genugel; Genugel CJ; Genugol RLV; Genuvisco J; Gum carrageenan; Gum chon 2; Gum chond; Gum Chrond; HSDB 1906; Irish moss; Irish moss extract; Irish moss gelose; Killeen; Lambda Carrageenan; Lygomme CDS; Marine Colloids Mixture of sulfated polysaccharides extracted from red seaweed (rhodophyceae); Mixture of sulphated polysaccharides extracted from red seaweed (rhodophyceae); Norsk gelatan; PC-515; Pearlpuss; Pellugel; Pellugel ID; Pencogel; Pig-Wrack; Plant material obtained from various members of the gigartinaceae or solieriaceae families of red seaweed; Satiagel GS350; Satiagum 3; Satiagum standard; Sea Spen; Seakem carrageenin; Self rock moss; Soa Ace WX 138; UNII-5C69YCD2YJ; Viscarin; Viscarin TP 389 (ChemIDplus).

1.3. Molecular formula

Unspecified (ChemIDplus).

1.4. Structural Formula

Not applicable.

1.5. Molecular weight (g/mol)

1,000-1,000,000 (commercial) (HSDB, 2002).

1.6. CAS registration number

9000-07-1

1.7. Properties

1.7.1. Melting point

(ºC): No data available to us at this time.

1.7.2. Boiling point

(ºC): No data available to us at this time.

1.7.3. Solubility

1 g dissolves in 100 mL water at about 80ºC (HSDB, 2002).

1.7.4. pKa

No data available to us at this time.

1.7.5. Flashpoint

(ºC): No data available to us at this time.

1.7.6. Flammability limits (vol/vol%)

No data available to us at this time.

1.7.7. (Auto)ignition temperature

(ºC): No data available to us at this time.

1.7.8. Decomposition temperature

(ºC): No data available to us at this time.

1.7.9. Stability

Solutions & gels fairly stable over wide pH range at room temp or lower; rapidly degraded at low pH & high temp; solutions at pH 9 are most stable (HSDB, 2002).

1.7.10. Vapor pressure

No data available to us at this time.

1.7.11. log Kow

No data available to us at this time.

2. General information

2.1. Exposure

Chondrus crispus powder (CAS RN 9000-07-1) is used as an abrasive ingredient in cosmetics in the EU.

Carrageenan (CAS RN 9000-07-1) is listed as an ingredient in Cosing database but without any mentioned function.

As taken from CosIng (undated).

Carrageenan (CAS RN 9000-07-1) is listed as an ingredient (at given concentrations, where specified) in inside the home (1.0-5.0%), personal care (0.1-5.0%) and pet care products by the CPID.

SURVEY OF FLAVORING INGREDIENT USAGE LEVELS

Flavoring Extract Manufacturer's Association average maximum levels (in ppm) on which the expert panel based its judgments that the substances are generally recognized as safe. Those substances which appear only on an FDA "White List" are indicated with an asterisk.

FEMA No. and Substace	No. of Reports	Beverages	Ice Cream, Ices Etc.	Candy	Baked Goods	Gelatins and Puddings	-Other Category Uses-	
2596 *IRISH MOSS, EXTRACT [Chondrus crispus (L.) Stackh. or Gigartina mamillosa (Gooden. et Woodw.) J. Ag.]--Carrageen, extract; Chondrus, extract	24	300.	390.	3.0 500.	1,3000.	1,700. 20,000.	Jellies (1)200.	Syrups 1,300.

1. Provided it is used at levels such that no thujone is detectable in the finished food, using the standard AOAC method.

As taken from Hall and Oser, 1965.

“Carrageenan is used in pharmaceutical products as well-known excipient, e.g. as pseudo-emulsifier or as thickening and gelling agent (Teuscher, 1997). It is used in solid, semi-solid and liquid dosage forms (Blaschek et al., 2016).

In former times, carrageenan was used as mucilage in cases of diarrhoea and as bulk-foaming laxative to treat obstipation. The dosage was given with 1.5 g herbal substance/cup of water as decoction. Gastrointestinal discomfort including gas in the intestine is mentioned as possible undesired effect when carrageenan is taken in very high amounts, as for all bulk forming substances (Blaschek et al., 2014; Martindale, 2014).

Carrageenan is used in some countries in combination products (Martindale, 2014).

For carrageenan as an active ingredient, only few authorised medicinal combination products exist within the EU but not for oral use.”

As taken from EFSA, 2018.

“Reported uses (from use as a food flavouring) ppm: (FEMA, 1994)

Food category	**Usual**	**Max.**	**Food category**	**Usual**	**Max.**
Alcoholic beverages	1.00	3.00	Imitation dairy	0.62	1.16
Baked goods	0.61	2.61	Jams, jellies	7.59	11.27
Cheese	0.05	0.06	Milk products	0.63	0.96
Condiments, relishes	1.00	4.98	Nonalcoholic beverages	0.28	0.41
Confection, frosting	2.00	2.00	Poultry	1.20	1.20
Frozen dairy	0.14	0.25	Soft candy	0.50	1.00

Fruit juice	0.50	1.00	Soups	0.20	0.20
Gelatins, puddings	2.03	4.53	Sweet sauce	2.03	3.87

Consumption (from use as a food flavouring), individual: 0.7937 mg/kg /day."

As taken from Burdock, 2010.

Carrageenan (CAS RN 9000-07-1) is used as a binder, and emulsifying, gelling, stabilizing, thickening and viscosity increasing agents in non-medicinal natural health products (Health Canada, 2021).

2.2. Combustion products

No data available to us at this time.

2.3. Ingredient(s) from which it originates

Carrageenan is the plant material obtained from various members of the Gigartinaceae or Solieriaceae families of the red seaweed, Rodophyceae.

Chondrus Crispus Powder is (carrageenan) the powder obtained from the dried, ground Alga, Chondrus crispus.

As taken from CosIng (undated)

"Native carrageenan is defined as a hydrocolloid isolated from red algae (seaweed)" (CIR, 2015). "Carrageenan is a generic term used for chemically similar hydrocolloids obtained by aqueous extraction from certain members of the class Rhodophyceae." As taken from Burdock, 2010

"A water-soluble extractive mixture of sulfated polysaccharides from RED ALGAE. Chief sources are the Irish moss CHONDRUS CRISPUS (Carrageen), and Gigartina stellata. It is used as a stabilizer, for suspending COCOA in chocolate manufacture, and to clarify BEVERAGES."

As taken from ChemIDplus.

3. Status in legislation and other official guidance

Carrageenan (CAS RN 9000-07-1) has been identified as "low concern to human health by application of expert validated rules under the NICNAS targeted tier I approach" and "poses no unreasonable risk to human health based on Tier I assessment under the NICNAS IMAP assessment framework" (AICIS, 2017).

Summary of Evaluations Performed by the Joint FAO/WHO Expert Committee on Food Additives

CARRAGEENAN	
General information	
Synonyms:	CARRAGEENAN (FROM CHONDRUS AND GIGARTINA SPP.), DANISH AGAR (FROM FURCELLARIA FASTIGIATA), EUCHEUMAN (FROM EUCHEUMA SPP.), FURCELLARAN (FROM FURCELLARIA FASTIGIATA), HYPNEAN (FROM HYPNEA SPP.), IRIDOPHYCAN (FROM IRIDAEA SPP.), IRISH MOSS GELOSE (FROM CHONDRUS SSP.)

CAS number:	9000-07-1
INS:	407
Functional Class:	· Food Additives o EMULSIFIER o GELLING_AGENT o STABILIZER o THICKENER
Evaluations	
Evaluation year:	**2014**
ADI:	NOT SPECIFIED
Comments:	The Committee reviewed data published since the sixty-eighth meeting in 2007, focusing in particular on data of relevance to the safety assessment of the use of carrageenan (but not processed Eucheuma seaweed) in infant formula. [...] The Committee concluded that the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1000 mg/L is not of concern. The Committee recognized that there is variability in medical conditions among infants requiring formulas for special medical purposes that contain the higher levels of carrageenan, and the Committee noted that these infants would normally be under medical supervision.
Meeting:	79
Specs Code:	R
Report:	TRS 990-JECFA 79/14
Specification:	FAO JECFA Monographs 16/7
Addendum Name:	FAS 70-JECFA 79/13
Evaluation year:	**2007**
ADI:	NOT SPECIFIED
Comments:	Group ADI for carrageenan and processed eucheuma seaweed. The Committee was of the view that based on the information available, it is inadvisable to use carrageenan or processed eucheuma seaweed in infant formulas.
Meeting:	28
Specs Code:	R (1985)
Report:	TRS 947-JECFA68/
Tox	FAS 59-JECFA68/

Monograph:	
Specification:	FAO JECFA Monographs 4- JECFA 68/ . R
Previous Years:	2001, TRS 909-JECFA 57/32, COMPENDIUM ADDENDUM 9/FNP 52 Add.9/13; FAO JECFA Monographs 1 vol.1/341, FAS 48-JECFA 57/91. NOT SPECIFIED (GROUP ADI FOR CARRAGEENAN AND PROCESSED EUCHEUMA SEAWEED). NS. R 1998, TRS 891-JECFA 51/37, COMPENDIUM ADDENDUM 6/FNP 5

As taken from WHO, 2022.

Carrageenan (CAS RN 9000-07-1) is listed as an approved ingredient in food and non-food use pesticide products by the US EPA InertFinder Database (2022)

For food use, it is regulated under 40 CFR Part 180.910 (Inert ingredients used pre- and post-harvest; exemptions from the requirement of a tolerance; “Limits: Minimum molecular weight (in amu): 100,000”; use: thickener) 180.920 (Inert ingredients used pre-harvest; exemptions for the requirement of a tolerance; “Limits: Not more than 0.15% of pesticide formulation”; use: thickener and stabilizer for pesticide formulations applied to seeds before planting) and 180.930 (Inert ingredients applied to animals; exemptions from the requirement of a tolerance; “Limits: Minimum molecular weight (in amu): 100,000”; use: thickener). (US EPA, 2022)Irish moss extract (CAS RN 9000-07-1) is listed as fragrance ingredient in the IFRA transparency list (IFRA, undated).

IARC Classification Group 3 - Not classifiable as to its carcinogenicity to humans. Volume 31, Sup 7, 1987.

As taken from IARC, 2022.

Carrageenan is included on the FDA’s inventory of “Substances Added to Food (formerly EAFUS)” as an anticaking agent or free-flow agent, drying agent, emulsifier or emulsifier salt, flavor enhancer, flavoring agent or adjuvant, formulation aid, humectant, processing aid, stabilizer or thickener and texturizer, and is included under 21 CFR sections 133.178 (Pasteurized neufchatel cheese spread with other foods), 133.179 (Pasteurized process cheese spread), 136.110 (Bread, rolls, and buns), 139.121 (Nonfat milk macaroni products), 139.122 (Enriched nonfat milk macaroni products), 172.620 (Food additives permitted for direct addition to food for human consumption – gums, chewing gum bases and related substances - carrageenan) and 182.7255 (Substances generally recognized as safe – stabilizers – Chondrus extract) (FDA, 2022a,b).

“Environmental Standards & Regulations:

FIFRA Requirements:

Residues of Carrageenan conforming to 21 CFR 172.620 (minimum molecular weight: 100,000) are exempted from the requirement of a tolerance when used as a thickner in accordance with good agricultural practices as inert (or occasionally active) ingredients in pesticide formulations applied to growing crops or to raw agricultural commodities after harvest. [40 CFR 180.1001(c) (7/1/99)] **PEER REVIEWED**

Carrageenan conforming to 21 CFR 172.620 (not more than 0.15% of pesticide formulation) is exempted from the requirement of a tolerance when used as a thickener and stabilizer for pesticide formulation applied to seeds before planting in accordance with good agricultural practice as inert (or occasionally active) ingredients in pesticide formulations applied to growing crops only. [40 CFR 180.1001(d) (7/1/99)] **PEER REVIEWED**

Carrageenan conforming to 21 CFR 172.620 (minimum molecular weight: 100,000) is exempted from the requirement of a tolerance when used as a thickener in accordance with good agricultural practice as inert (or occasionally active) ingredients in pesticide formulations applied to animals. [40 CFR 180.1001(e) (7/1/99)] **PEER REVIEWED**

Acceptable Daily Intakes:

FOOD & AGRICULTURE ORGANIZATION OF UNITED NATIONS/WORLD HEALTH ORGANIZATION ACCEPTABLE DAILY INTAKE: 0-75 MG/KG BODY WT. /FROM TABLE/ [Doull, J., C.D. Klaassen, and M. D. Amdur (eds.). Casarett and Doull's Toxicology. 2nd ed. New York: Macmillan Publishing Co., 1980., p. 600] **PEER REVIEWED**

FDA Requirements:

Carrageenan is a food additive permitted for direct addition to food for human consumption, as long as 1) the quantity of the substance added to food does not exceed the amount reasonably required to accomplish its intended physical, nutritive, or other technical effect in food, and 2) any substance intended for use in or on food is of appropriate food grade and is prepared and handled as a food ingredient. [21 CFR 172.620 (4/1/99)] **PEER REVIEWED**

Carrageenan with polysorbate 80 is a food additive permitted for direct addition to food for human consumption, as long as 1) the quantity of the substance added to food does not exceed the amount reasonably required to accomplish its intended physical, nutritive, or other technical effect in food, and 2) any substance intended for use in or on food is of appropriate food grade and is prepared and handled as a food ingredient. /Carrageenan with polysorbate 80/ [21 CFR 172.623 (4/1/99)] **PEER REVIEWED**

Salts of carrageenan is a food additive permitted for direct addition to food for human consumption, as long as 1) the quantity of the substance added to food does not exceed the amount reasonably required to accomplish its intended physical, nutritive, or other technical effect in food, and 2) any substance intended for use in or on food is of appropriate food grade and is prepared and handled as a food ingredient. /Salts of carrageenan/ [21 CFR 172.626 (4/1/99)] **PEER REVIEWED**

Chondrus extract (carrageenin) is generally recognized as safe when used in accordance with good manufacturing practice. [21 CFR 182.7255 (4/1/99)] **PEER REVIEWED**

Manufacturers, packers, and distributors of drug and drug products for human use are responsible for complying with the labeling, certification, and usage requirements as prescribed by the Federal Food, Drug, and Cosmetic Act, as amended (secs 201-902, 52 Stat. 1040 et seq., as amended; 21 U.S.C. 321-392). [21 CFR 200-299, 300-499, 820, and 860 (4/1/99)] **PEER REVIEWED**"

As taken from HSDB, 2002

Carrageenan is listed on the Safer Chemical Ingredients List (US EPA, 2022).

Carrageenan (CAS RN 9000-07-1) is listed on the US EPA Toxic Substances Control Act (TSCA) inventory, US EPA 2020 CDR Full Exempt list (Chemical Data Reporting Rule) and 2020 CDR TSCA Inv Active and also on the TSCA Exemption under CDR (flag XU) list.

Not registered under REACH (ECHA).

Carrageenan (CAS RN 9000-07-1) is not classified for packaging and labelling under Regulation (EC) No. 1272/2008 (ECHA, 2022).

Carrageenan (E 407) is authorised for use as a food additive under EU regulation nos 1129/2011 and 0601/2014, and also as a Group I additive under EU regulation nos 1129/2011, 438/2013, 2015/0647, 2015/1832 and 2018/1497 (European Commission, 2015, 2019).

Irish Moss Extract (Chondrus crispus (L.) Stackh. or Gigartina mamillosa (Gooden. et Woodw.) J. Ag.) (CAS RN 9000-07-1; FEMA no. 2596) has been designated as GRAS (generally recognized as safe) by FEMA (Hall and Oser, 1965).

“Regulatory status CoE: n/a FDA: 21 CFR et seq.; 136.110; 139 et seq.; 150 et seq.; 172.620; 172.623; 172.626; 201.319; 27 CFR 24.243 FDA (other): CDER

JECFA: ADI: Not specified (group ADI).”

As taken from Burdock, 2010.

Carrageenan is included on the US FDA’s list of inactive ingredients for approved drug products. It is permitted for use as an ingredient in various products, at the following maximum potencies per unit dose and maximum daily exposure:

Inactive Ingredient	Route	Dosage Form	CAS Number	UNII	Maximum Potency per unit dose	Maximum daily exposure (MDE)
CARRAGEENAN	DENTAL	PASTE	9000071	5C69YCD2YJ	0.3%w/w	
CARRAGEENAN	NASAL	POWDER	9000071	5C69YCD2YJ	NA	
CARRAGEENAN	ORAL	CAPSULE	9000071	5C69YCD2YJ		3mg
CARRAGEENAN	ORAL	CAPSULE, DELAYED RELEASE	9000071	5C69YCD2YJ		1mg
CARRAGEENAN	ORAL	GRANULE, FOR SUSPENSION	9000071	5C69YCD2YJ		161mg
CARRAGEENAN	ORAL	POWDER	9000071	5C69YCD2YJ		1mg
CARRAGEENAN	ORAL	POWDER, FOR SUSPENSION	9000071	5C69YCD2YJ	15mg	
CARRAGEENAN	ORAL	SUSPENSION	9000071	5C69YCD2YJ	6mg/ 5ml	
CARRAGEENAN	ORAL	TABLET	9000071	5C69YCD2YJ		15mg
CARRAGEENAN	RESPIRATORY (INHALATION)	POWDER	9000071	5C69YCD2YJ	0.42mg	
CARRAGEENAN	TOPICAL	LOTION	9000071	5C69YCD2YJ	0.5%w/w	
CARRAGEENAN	TRANSDERMAL	FILM, EXTENDED RELEASE	9000071	5C69YCD2YJ	33mg	

As taken from FDA, 2022c

Carrageenan (CAS RN 9000-07-1) is classified as a natural health product (NHP) under Schedule 1, item 2 (an isolate) of the Natural Health Products Regulations (Health Canada, 2021).

4. Metabolism/Pharmacokinetics

4.1. Metabolism/metabolites

“A total of 154 strains from 22 species of Bifidobacterium, Peptostreptococcus, Lactobacillus, Ruminococcus, Coprococcus, Eubacterium and Fusobacterium, which occur in high concentrations in the human colon, were tested for their ability to ferment 21 different complex carbohydrates. Among them, carrageenan (unspecified type; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30) was not fermented by any strains of the investigated species (Salyers et al., 1977).

Fermentations of 10 polysaccharides including carrageenan (unspecified type; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30), by 24 species of the family Enterobacteriaceae (Klebsielleae and other gram-negative facultative bacilli) were examined by Ochuba and von Riesen (1980). Carrageenan was fermented by 15 species including Klebsiella (K. oxytoca and K. pneumoniae), Aeromonas, Enterobacter, Pectobacterium, Serratia and Vibrio strains. According to the authors, this study demonstrated the possible fermentation of carrageenan by enteric bacteria.”

As taken from EFSA, 2018.

4.2. Absorption, distribution and excretion

“In groups of three to seven male and female rats (no further information about the strain) fed with 2, 5, 10, 15, or 20% (about 2,000–20,000 mg/kg bw per day) of κ/λ-carrageenan from C. crispus (commercial product – Seakem, type 6, from Marine Colloids, Rockland, Maine; interpreted by the Panel as isolated from C. crispus with an approximate ratio ι:κ:λ type 0:70:30, ‘molecular weight distribution not indicated’) over 23–148 days via the diet, more than 90% of the ingested carrageenan was excreted via faeces (Hawkins and Yaphe, 1965).”

“Studies conducted with Sprague–Dawley rats fed diets containing 4% κ/λ-carrageenan (commercial product calcium carrageenan, Sea Kem, type II, Marine Colloids, Co.; interpreted by the Panel as C. crispus with an approximate ratio ι:κ:λ type 0:70:30,‘molecular weight distribution not indicated’), separate absorption studies were done with adult males (Tomarelli et al., 1974). The animals (n=8) were placed in metabolism cages, fed a basal diet for 7 days, followed by 5 days of feeding the carrageenan diet, and then 2 more days on the basal diet. By using an improved colorimetric method, 99% of the ingested carrageenan was found to be excreted via faeces.”

“In another experiment weanling Sprague–Dawley rats of both sexes (n = 5) were fed control diet or a diet containing 5% (about 5,000 mg/kg bw per day) of carrageenan from C. crispus (number-average molecular weight: 186–214 kDa ratio ι:κ:λ types 0:71:29 to 0:88:12, Stauffer Chemicals, Inc). After 13 weeks, five animals of each group and sex were killed and samples of liver were analysed for carrageenan. Faecal samples were also collected for analysis. Faecal and liver samples were examined qualitatively by gel electrophoresis, and quantitative measurements of carrageenan were carried out on samples of liver and urine. For the high molecular carrageenan (>100 kDa) no or only little absorption was noted based on the absence of carrageenan from the livers of rats.”

“Chen et al. (1981) used a gel electrophoresis method developed for the detection of carrageenan (unspecified type; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30, ‘molecular weight distribution not indicated’) in rat liver homogenates

(measurable limit of 15 µg carrageenan/g liver tissue). No carrageenan was found in the livers of rats fed 25% (about 25,000 mg/kg bw per day) over one month. Further details were not reported.

In the subchronic study of Abraham et al. (1985), three groups of 30 Sprague–Dawley rats were exposed to 1% or 5% carrageenan (commercial product Iridaea carrageenan from Hercules Inc., Copenhagen Pectin Fabrik Ltd., Denmark; interpreted by the Panel as isolated from Iridaea ciliate = Sarcothalia crispata, mixture of κ and λ,'molecular weight distribution not indicated') via diet (equivalent to 900 and 4,500 mg/kg bw per day or with cellulose at a 5% dietary level (13 males and 17 females) for 13 weeks. Storage of carrageenan or carrageenan-like material was investigated by the presence of metachromatic material following toluidine blue staining, increase in lysosomal enzyme activity in sites similar to ultrastructural localisation of fibrillar material and chemical analysis. According to the authors, this study showed no retention or storage of native carrageenan in the gut or liver of chronically administered rats. The absence of tissue retention of native carrageenan was also observed in an additional chronic experiment in which two strains of rats received this carrageenan for 39 weeks."

"Other groups of female guinea pigs (n=3) were given by gavage, carrageenan from …..C. crispus (number-average molecular weight of 8.5–314 kDa, ratio ι:κ:λ types 0:100;0; 0:0:100 or 0:70:30, Marine Colloids Inc.) as 1% solutions (about 1,000–2,000 mg/kg bw per day) via drinking water for 2 weeks. At the end of the…..experiment samples of liver and faeces were examined. In all trials, faecal and liver samples were examined qualitatively by gel electrophoresis, and quantitative measurements of carrageenan were carried out on samples of liver and urine. For the high molecular weight carrageenan (>100 kDa), no or only little absorption was noted based on the absence of carrageenan in the livers or the urine of guinea pigs. By contrast, significant amounts of carrageenan were found in livers of animals given carrageenan with number-average molecular weight lower than 88 kDa, whatever the type of carrageenan was. According to the authors, urinary excretion of carrageenan was limited to low-molecular-weight material (number-average molecular weight: <20 kDa). Qualitative and quantitative evidence indicated that there was an upper limit to the size of carrageenan molecules absorbed, but estimates of this upper limit ranged from 10 to 85 kDa depending upon the analytical approach. Absorption of low molecular carrageenan from the drinking-water was generally higher than following dietary administration. The Panel noted that low molecular carrageenan would be partly absorbed, particularly when administered by gavage and given via drinking water."

"The studies in neonatal minipigs and in neonatal pig (Documentation provided to EFSA n. 32; Documentation provided to EFSA n. 33; also described in Weiner et al., 2015) employed a liquid chromatography with tandem mass spectrometry (LC–MS/MS) analytical method developed using 'poligeenan' as a surrogate for detection of the low molecular weight carrageenan in blood.

In the neonatal minipig study (Gottingen minipigs) (Documentation provided to EFSA n. 32), following daily oral administration of carrageenan (the test compound is assumed to be identical with that described below in the study by Weiner et al., 2015) at doses of 0, 300 or 3,000 mg/kg formula (equal to 60–66 or 600–666 mg/kg bw per day) for 10 days, starting on postnatal day (PND) 2, there were no quantifiable serum poligeenan concentrations in animals receiving the 600–666 mg/kg bw per day on both evaluation days. According to the authors, the unique reported values for two animals (one control and one receiving 60–66 mg/kg bw per day) may represent interfering compounds rather than actual exposure to low molecular weight carrageenan.

In the study in neonatal pigs (Domestic Yorkshire Crossbred Swine) (Documentation provided to EFSA n. 33; Weiner et al., 2015), the test material sample of κ/λ-carrageenan (FMC Lot. 90303011) (ratio κ/λ unknown) was fully characterised for molecular weight, percentage of molecular weight below 50 kDa, known as LMT as per European requirements. The weight-average molecular weight of the test material sample was 664–732 kDa with a LMT of 0.3–3.9%. The test substance was administered at doses of 0, 300, 1,000 or 2,250 mg/kg formula for 28 days starting on PND 3 (highest dose equal to 430–448 mg/kg bw per day). The authors reported an analytical signal in male piglets, corresponding to that expected for the low molecular weight carrageenan. According

to the author, this pattern was not consistent with the expected exposure based on the calculated compound consumption of carrageenan. This is supported by the fact that (1) a positive signal was elicited in all males in the control group; (2) similar values were observed in all treated males at 300, 1,000, and 2,250 mg/kg; (3) the signal did not produce a dose response effect in the treated groups (300, 1,000 and 2,250 mg/kg) and (4) a positive signal was almost exclusively limited to male animals. These data would indicate that the signal in the plasma samples is not a low molecular carrageenan.

Considering the study in neonatal minipigs and neonatal pigs, the Panel noted the use of appropriate animal models for representing the immature gastrointestinal tract in human infants. However, due to problems in the outcomes of analytical assays, the Panel was unable to conclude on a possible in vivo degradation of carrageenan."

"In the short-term study of Abraham et al. (1972), male and female rhesus monkeys (Macaca mulatta, n = 10) were given a type of κ/λ-carrageenan derived from C. crispus (carrageenan mixture of κ and λ with number-average molecular weight of approx. 800 kDa obtained from Marine Colloids, Inc. Rockland, ME, Lot. Beta282400; interpreted by the Panel as C. crispus with an approximate ratio ι:κ:λ type 0:70:30) via drinking water as a 1% solution (about 1,300 mg/kg bw per day) for 7–11 weeks. There was no evidence of carrageenan storage….."

"In the long-term toxicity study (Documentation provided to EFSA n. 34), rhesus monkeys (19 males and 21 females) were divided into four groups with 10 animals each and dosed with 0, 50, 200, 500 mg/kg bw per day κ/λ-carrageenan (commercial product Gelcarin HMR from Marine Colloids, Rockland, Maine; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30, molecular weight distribution not indicated) by gavage daily on 6 days a week for the first 5 years, whereas for the remaining 2.5 years, carrageenan was incorporated into diet and given to monkeys. In this study, there was also no evidence for the uptake of fibrillar, metachromic, carrageenan-like material in the cells of the reticular-endothelial system in the liver, lymph nodes, caecum, colon or anorectal junction.

In the study of Pittman et al. (1976), rhesus monkeys of both sexes (n = 2) were given carrageenan from C. crispus (number-average molecular weight: 185 kDa, ratio ι:κ:λ type 0:70:30, Marine Colloids Inc.), daily in doses of 50, 200 or 500 mg/kg bw per day by gavage. Overnight collections of faeces were obtained after 11 months. Overnight collections of urine were obtained from two of the monkeys on the high dose after 15 months….. In animals receiving carrageenan of number-average molecular weight: 185 kDa no or only traces of carrageenan were excreted in urine."

"Overall, the Panel noted the absence of metabolic studies of carrageenan in humans. In several in vivo studies in rats, guinea pigs, neonatal minipigs, neonatal pigs and monkeys, faecal excretion of carrageenan was shown to be 98 – 100% of the ingested amount. These studies included rats with normal gut microflora and rats with their gut microflora replaced by human faecal microflora. Several short-term and long-term studies performed in rats dosed via diet containing different types of carrageenan confirmed the absence of tissue storage of this carrageenan."

"The Panel noted that the conditions (duration, pH, temperature) for potential hydrolysis of carrageenan in the GI tract are less extreme than the 'mild' acidic conditions which resulted in the formation of chemically degraded carrageenan (C16). The theoretical possibility that more limited degradation could occur under conditions representative of the in vivo situation remains an uncertainty although this has not been observed to any significant extent in the in vivo ADME studies reported."

As taken from EFSA, 2018.

"Carrageenan is not degraded or absorbed in the gastrointestinal tracts of rodents, dogs, and non-human primates."

As taken from CIR, 2015.

Previous study has suggested the colonic nontoxicity and obesity inhibition of food-grade κ-carrageenan in obese mice. Further study using transcriptome is important to provide further understanding on the gene expressions of inflammation and obesity. Here, the obese mice without any treatment (HFD) or with 5% food-grade κ-carrageenan diet intervention (H5%) were used to perform colonic transcriptome sequencing. The results showed that genes involved in the inflammatory pathways or tight junction protein encoding were not significantly dysregulated by 5% carrageenan. However, the expression of lipid metabolism genes meaningfully changed as evidenced by the decreased gene levels of adipocytokines, lipogenesis, lipid absorption and transport, and the increased adipolysis and oxidation. In addition, the carrageenan metabolism experiments by toluidine blue (TB) staining of colon and high-performance size exclusion chromatography (HPSEC) of feces supernatant showed that the food-grade κ-carrageenan was not absorbed or significantly degraded in the digestive tract of obese mice. Hence, the fact that food-grade κ-carrageenan was not significantly metabolized by the organism and did not cause obvious dysregulation of colonic inflammatory genes provided evidences for its noncolonic toxicity in obese mice. An anti-obesity potential of food-grade κ-carrageenan was probably mediated by the regulation of lipids metabolism-related genes.

As taken from Zhang H et al. 2021

4.3. Interactions

“Harmuth-Hoene and Schelenz (1980) investigated the effect of polysaccharides, including carrageenan (unspecified type; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30; Copenhagen Pectin Factory Ltd), given at the 10% level in a semi-synthetic diet (equivalent to 1,200 mg/kg bw per day), on absorption of Ca, Fe, Zn, Cu, Cr and Co, on weight gain and on faecal dry matter excretion, in five groups of 12 weanling male rats each and compared to a control group, over a period of 8 days. No indication on weight-average molecular weight was given. Carrageenan reduced significantly the absorption of all minerals tested and increased both their faecal excretion and that of faecal dry matter.”

As taken from EFSA, 2018.

“The inhibitory effect of carrageenan-induced inflammation was examined by utilizing rats treated with inducers of this drug metabolizing system. Animals were given sodium phenobarbital (PB, 80 mg/kg, i.p., 24 h prior to death), or benzo[a]pyrene (BP, 40 mg/kg, i.p., 24 h prior to death) as inducers. Some animals were also given carrageenan 24 h prior to death. Non-induced male rats exhibited significant decreases in hepatic 9000 x g supernatant (S-9) cytochrome P-450 and aminopyrine (AM) N-demethylase, benzphetamine (BenzP) N-demethylase and meperidine (MP) N-demethylase activity following carrageenan treatment. Carrageenan also depressed the induction of hepatic S-9 cytochrome P-450 content caused by PB treatment, and suppressed the induction of AM, BenzP, MP, arylhydrocarbon and 7-ethoxycoumarine metabolism by PB treatment. Cytochrome P-450 levels and related biotransformation activity which are elevated by BP treatment were not decreased by the injection of BP and carrageenan simultaneously to male rats. Non-induced, PB-treated and BP-treated female rats did not show inhibited carrageenan-induced reduction in hemoprotein content or inhibition of AM-N-demethylase, BenzP N-demethylase and aniline hydroxylase activities. These results demonstrate the selective nature of the inhibitory effects of carrageenan-induced inflammation upon drug metabolism in the rats.” As taken from Ishikawa M et al. 1992a. J. Pharmacobiodyn. 15(4), 139-146. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/1494976

“Following in vivo treatment with carrageenan, sex-related differences in alteration of hepatic drug metabolism were found in the rat. In adult male rats, marked decreases were observed in hepatic 9000 x g supernatant cytochrome P-450 content and in the biotransformation of hexobarbital, aminopyrine, ethylmorphine, and meperidine. Hexobarbital hypnosis was significantly prolonged by

carrageenan treatment in intact and testectomized animals as compared to their respective controls. Although carrageenan-treated intact animals slept 480% longer, carrageenan-treated testectomized rats slept only 60% longer than the respective control animals. However, testectomy or administration of 17 beta-estradiol to testectomized male rats did not inhibit the monooxygenase activities by carrageenan-treatment. Furthermore, administration of testosterone to ovariectomized female rats did not antagonize the inhibitory effects of the carrageenan-induced inflammation. The inhibitory effects produced by carrageenan-induced inflammation on the microsomal enzyme system were observed only in mature male rats and were not observed in mature female rats or in sexually immature rats of either sex. Thus, these results suggest that the inhibitory effects of carrageenan-induced inflammation on hepatic 9000 x g supernatant monooxygenases in the male rat are partially mediated through the toxic action of carrageenan-induced inflammation on androgen-dependent factors in this enzyme system." As taken from Ishikawa M et al. 1992b. J. Pharmacobiodyn. 15(8), 367-376. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/1479536

"Effect of carrageenan-induced inflammation on hepatic drug metabolism was studied in male and female Wistar rats. One day after treatment of male and female Wistar rats with carrageenan (1%, 0.1 ml, s.c.) (i) a prolongation of the duration of sleeping time induced by hexobarbital (100 mg/kg, i.p.), (ii) decreases biotransformation of hexobarbital, aminopyrine and ethylmorphine in the hepatic 9000 x g supernatants (S-9) and (iii) a decrease in cytochrome P-450 content were observed in males but not female rats. However, carrageenan treatment did not alter aniline hydroxylase activity in animals of either sex. Carrageenan also suppressed induction of hexobarbital, aminopyrine and ethylmorphine metabolism by phenobarbital treatment, and depressed the induction of hepatic S-9 cytochrome P-450 content caused by phenobarbital treatment. These data show that there is a sex-related difference in the ability of carrageenan to alter hepatic drug metabolism, which is substrate-specific, in the rats." As taken from Ishikawa M et al. 1991. J. Pharmacobiodyn. 14(3), 132-138. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/1880703

"There is a growing body of evidence that implicates oxygen free radicals in a wide variety of inflammatory conditions in various body systems including the gastrointestinal tract. The purpose of this study was to ascertain whether or not oxy-radicals play a role in carrageenan-mediated intestinal injury. Allopurinol, superoxide dismutase-polyethylene glycol, and dimethyl sulfoxide, respectively, were administered to the carrageenan rat model for 30 to 32 days. Collectively, all three drugs attenuated the carrageenan-mediated injury as shown by four indices of intestinal damage: ulceration ($p = 0.0007$); abnormal villous pattern ($p = 0.0002$); degree of inflammation ($p = 0.0001$); and extent of inflammation ($p = 0.0025$). Dimethyl sulfoxide appeared to be the least efficacious of the three drugs. The results suggest that oxygen free radicals play a role in carrageenan-mediated intestinal injury, and that one of the sources of these oxy-radicals may be the intestinal macrophage." As taken from Moyana T and Lalonde JM. 1991. Ann. Clin. Lab. Sci. 21(4), 258-263. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/1859164

"Twenty-four hours after treatment with carrageenan the duration of hexobarbital-induced hypnosis was prolonged in males but not females in Wistar rats bearing carrageenan-induced paw edema. Similar treatment with carrageenan led to significant inhibition of the metabolism of hexobarbital by hepatic enzymes obtained from male but not female animals. These data suggest that there is a sex-related difference in the ability of carrageenan to alter drug actions in rats." As taken from Ishikawa M et al. 1989. Res. Commun. Chem. Pathol. Pharmacol. 65(2), 261-264. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/2587845

"The urinary excretion of two orally administered polyethylene glycol markers (PEG 900 and PEG 4000) was measured in rats after feeding various carbohydrate gelling agents. Pectin, guaran, methylcellulose, and carrageenan were added (20% wt/wt) to a fiber-free control diet and were fed for 4 wk prior to the experiment. Excretion of 3H-PEG 900 and 14C-PEG 4000 was measured over a period of 96 h after administration of the markers in the drinking water. In general, the ratio of PEG 4000 to PEG 900 in the urine increased after fiber feeding: from 0.20 in the controls to 0.31,

0.37, 0.29, or 0.27 (medians) in the pectin-, guaran-, methylcellulose-, or carrageenan-fed group, respectively. The pattern of excretion of the two PEG markers in rats fed the fiber-free diet differed from that in the polysaccharide-fed rats. Pectin and guaran, two polysaccharides readily fermented by intestinal bacteria, led to a higher excretion of PEG 4000 but an unaltered excretion of PEG 900, whereas methylcellulose and carrageenan, two polysaccharides more inert against microbial degradation, were associated with a lower excretion of PEG 900 with unchanged excretion of PEG 4000. The study shows that polysaccharides, which may be representative of the soluble portion of dietary fiber, can influence the intestinal permeability of larger molecules. The microbiologically degradable polysaccharides in particular may lead to an increased absorption of larger molecules." As taken from Elsenhans B et al. 1989. J. Nutr. 119(3), 380-387. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/2537888

5. Toxicity

5.1. Single dose toxicity

Type of Test	Route of Exposure or Administration	Species/Test System	Dose Data	Toxic Effects	Reference
LDLo - Lowest published lethal dose	Intravenous	Rodent - rabbit	5 mg/kg	Blood - change in clotting factors	JPPMAB Journal of Pharmacy and Pharmacology. (Pharmaceutical Soc. of Great Britain, 1 Lambeth High St., London SEI 7JN, UK) V.1- 1949- Volume(issue)/page/year: 17,647,1965
LDLo - Lowest published lethal dose	Intravenous	Rodent - guinea pig	20 mg/kg	Kidney/Ureter/Bladder - other changes	NATUAS Nature. (Nature Subscription Dept., POB 1018, Manasguan, NJ 08736) V.1- 1869- Volume(issue)/page/year: 202,401,1964
TDLo - Lowest published toxic dose	Intrapleural	Rodent - rat	16 mg/kg	Lungs, Thorax, or Respiration - pleural effusion Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	JPETAB Journal of Pharmacology and Experimental Therapeutics. (Williams & Wilkins Co., 428 E. Preston St., Baltimore, MD 21202) V.1- 1909/10- Volume(issue)/page/year: 299,519,2001
TDLo - Lowest published toxic	Subcutaneous	Rodent - rat	24 mg/kg	Spinal Cord - inflammatory changes Biochemical - Metabolism	EJPHAZ European Journal of Pharmacology. (Elsevier Science Pub. B.V., POB 211, 1000 AE

dose				(Intermediary) - other proteins Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	Amsterdam, Netherlands) V.1- 1967- Volume(issue)/page/year: 441,141,2002
TDLo - Lowest published toxic dose	Unreported	Rodent - mouse	20 ug/kg	Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation Gastrointestinal - other changes	FATOAO Farmakologiya i Toksikologiya (Moscow). For English translation, see PHTXA6 and RPTOAN. (V/O Mezhdunarodnaya Kniga, 113095 Moscow, USSR) V.2- 1939- Volume(issue)/page/year: 49,57,1986
TDLo - Lowest published toxic dose	Parenteral	Rodent - rat	4 mg/kg	Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	FATOAO Farmakologiya i Toksikologiya (Moscow). For English translation, see PHTXA6 and RPTOAN. (V/O Mezhdunarodnaya Kniga, 113095 Moscow, USSR) V.2- 1939- Volume(issue)/page/year: 47,44,1984
TDLo - Lowest published toxic dose	Subcutaneous	Rodent - rat	6.67 mg/kg	Sense Organs and Special Senses (Eye) - optic nerve neuropathy	FATOAO Farmakologiya i Toksikologiya (Moscow). For English translation, see PHTXA6 and RPTOAN. (V/O Mezhdunarodnaya Kniga, 113095 Moscow, USSR) V.2- 1939- Volume(issue)/page/year: 44,46,1982
TDLo - Lowest published toxic dose	Subcutaneous	Rodent - mouse	1.8 mg/kg	Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	CYLPDN Zhongguo Yaoli Xuebao. Acta Pharmacologica Sinica. Chinese Journal of Pharmacology. (China International Book Trading Corp., POB 2820, Beijing, Peop. Rep. China) V.1- 1980- Volume(issue)/page/year: 22,375,2001

TDLo - Lowest published toxic dose	Intraperitoneal	Rodent - rat	25 mg/kg	Gastrointestinal - peritonitis	CYLPDN Zhongguo Yaoli Xuebao. Acta Pharmacologica Sinica. Chinese Journal of Pharmacology. (China International Book Trading Corp., POB 2820, Beijing, Peop. Rep. China) V.1- 1980- Volume(issue)/page/year: 22,375,2001
TDLo - Lowest published toxic dose	Intrapleural	Rodent - rat	0.006 mg/kg	Lungs, Thorax, or Respiration - pleural effusion Lungs, Thorax, or Respiration - other changes Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	BCPCA6 Biochemical Pharmacology. (Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, NY 10523) V.1- 1958- Volume(issue)/page/year: 63,785,2002
TDLo - Lowest published toxic dose	Subcutaneous	Rodent - rat	526.3 ug/kg	Biochemical - Enzyme inhibition, induction, or change in blood or tissue levels - other oxidoreductases Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	EJPHAZ European Journal of Pharmacology. (Elsevier Science Pub. B.V., POB 211, 1000 AE Amsterdam, Netherlands) V.1- 1967- Volume(issue)/page/year: 540,175,2006
TDLo - Lowest published toxic dose	Intraperitoneal	Rodent - rat	526.3 ug/kg	Gastrointestinal - other changes Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	EJPHAZ European Journal of Pharmacology. (Elsevier Science Pub. B.V., POB 211, 1000 AE Amsterdam, Netherlands) V.1- 1967- Volume(issue)/page/year: 540,175,2006
TDLo - Lowest published toxic dose	Intradermal	Rodent - rat	1 ng/kg	Vascular - structural changes in vessels	EJPHAZ European Journal of Pharmacology. (Elsevier Science Pub. B.V., POB 211, 1000 AE Amsterdam, Netherlands) V.1- 1967-

					Volume(issue)/page/year: 534,258,2006
TDLo - Lowest published toxic dose	Intraperitoneal	Rodent - mouse	23809.52 ug/kg	Vascular - other changes Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	BJPCBM British Journal of Pharmacology. (Macmillan Press Ltd., Houndmills, Basingstoke, Hants. RG21 2XS, UK) V.34-1968- Volume(issue)/page/year: 148,1060,2006
TDLo - Lowest published toxic dose	Subcutaneous	Rodent - rat	22.22 mg/kg	Biochemical - Enzyme inhibition, induction, or change in blood or tissue levels - other oxidoreductases Biochemical - Enzyme inhibition, induction, or change in blood or tissue levels - other Enzymes Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	BJPCBM British Journal of Pharmacology. (Macmillan Press Ltd., Houndmills, Basingstoke, Hants. RG21 2XS, UK) V.34-1968- Volume(issue)/page/year: 145,141,2005
TDLo - Lowest published toxic dose	Subcutaneous	Rodent - rat	400 ug/kg	Vascular - other changes Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	FCLPH* Fundamental & Clinical Pharmacology (Oxford : Blackwell Science) 2001- Volume(issue)/page/year: 18(Suppl 1),79,2004
TDLo - Lowest published toxic dose	Subcutaneous	Rodent - rat	400 ug/kg	Biochemical - Metabolism (Intermediary) - other proteins Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	FCLPH* Fundamental & Clinical Pharmacology (Oxford : Blackwell Science) 2001- Volume(issue)/page/year: 18(Suppl 1),89,2004
TDLo - Lowest publishe	Subcutaneous	Rodent - rat	400 ug/kg	Biochemical - Metabolism (Intermediary) -	FTRPAE Fitoterapia. (Inverni della Beffa SpA, via Ripamonti, 99,

d toxic dose				effect on inflammation or mediation of inflammation	20141 Milan, Italy) V.18-1947- Volume(issue)/page/year: 74,559,2003
TDLo - Lowest published toxic dose	Intraperitoneal	Rodent - mouse	15000 ug/kg	Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	TOXIA6 Toxicon. (Pergamon Press Ltd., Headington Hill Hall, Oxford OX3 OBW, UK) V.1- 1962- Volume(issue)/page/year: 55,1100,2010
TDLo - Lowest published toxic dose	Intraperitoneal	Rodent - mouse	23.8 mg/kg	Gastrointestinal - peritonitis	JOETD7 Journal of Ethnopharmacology. (Elsevier Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1-1979- Volume(issue)/page/year: 153,435,2014
TDLo - Lowest published toxic dose	Intrapleural	Rodent - rat	10.5 mg/kg	Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	JOETD7 Journal of Ethnopharmacology. (Elsevier Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1-1979- Volume(issue)/page/year: 154,319,2014
TDLo - Lowest published toxic dose	Subcutaneous	Rodent - rat	1 mg/kg/2 H	Vascular - other changes	TOXIA6 Toxicon. (Pergamon Press Ltd., Headington Hill Hall, Oxford OX3 OBW, UK) V.1- 1962- Volume(issue)/page/year: 117,37,2016
TDLo - Lowest published toxic dose	Intrapleural	Rodent - mouse	32.8 mg/kg	Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	JOETD7 Journal of Ethnopharmacology. (Elsevier Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1-1979- Volume(issue)/page/year: 191,9,2016
TDLo - Lowest published toxic dose	Intrapleural	Rodent - rat	8.3 mg/kg	Lungs, Thorax, or Respiration - acute pulmonary edema Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of	JOETD7 Journal of Ethnopharmacology. (Elsevier Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1-1979- Volume(issue)/page/year: 194,580,2016

				inflammation	

As taken from RTECS, 2018.

5.2. Repeated dose toxicity

“Groups of six male albino rats (no further information about the strain; initial body weights of 48 – 58 g) were dosed with 0%, 5%, 10% or 20% κ/λ-carrageenan (interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30) (equivalent to 0, 5,00, 11,800 or 23,600 mg/kg bw per day) via the diet for 10 weeks (Nilson and Schaller, 1941). No indication about weight-average molecular weight was given. Although treated rats showed more laxative condition, in general the animals grew well. Rats dosed with 20% required significantly more feed and water per gram of weight gain than the control group, and in this group, the weight gain also was about 30% less compared with controls. Three of six rats in this dose group died within 14 days after initiation (most probably due to a change in the bacterial flora), but the necropsies were essentially negative. Other parameters were not examined.”

“Rats (3–4 weeks old, strain not specified) were divided into groups of 10 males and 10 females, dosed for 50 days with carrageenan (commercial product Carasty-S, from the South Portland Maine Plant (USA), food ingredient product lot no. 10036; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30) via diet at dose levels of 0%, 15% or 25% (equivalent to 0, 18,000 or 30,000 mg/kg bw per day) (Documentation provided to EFSA n. 36). No indication about weight-average molecular weight was given. Body weights were recorded twice weekly and at final sacrifice on day 50. An interim sacrifice was done on day 31 (5 males and 5 females from each group) and at gross examination special attention was given to the liver. The rate of feed consumption for the test animals was generally less than the controls, resulting in a decreased weight gain especially in the early part of the study. In all dosed animals diarrhoea was noted after 4 days in study, which continued throughout the remaining period of the study, and the symptoms were more pronounced at a dietary level of 25%. Only at this dose level, male and female rats also excreted bloody stools. In addition, animals receiving carrageenan started losing hair from the dorsal mid-line surfaces on the back during days 8–50, and this finding was more severe in females of the 25% group. At interim sacrifice, it was noted that dosed animals appeared smaller compared with controls and also internal organs were correspondingly smaller. This effect was most prominent in males dosed with 25%. The livers of all animals were normal in texture and surface appearance, and no histopathological differences caused by dosing with carrageenan were seen. At terminal sacrifice on day 50, no gross pathological abnormalities were noted. Many of the livers examined had a mottled appearance, but this finding also occurred in controls. A histopathological examination of the livers gave no indication of adverse effects in all dose groups. Detailed statistics were not reported in this study.”

“Rhesus monkeys (M. mulatta; 3 males and 3 females) were given solutions of 1% carrageenan composed of κ- and λ-carrageenan (commercial product from Marine Colloids, Inc., Rockland; lot n. 282400, number-average molecular weight of 800 kD; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30)….. in drinking water over a period of 7 – 11 weeks, followed by recovery for up to 24 weeks (Abraham, 1972). There was no evidence of storage of carrageenan,….. Ultrastructurally, the hepatocytes appeared normal in all groups…..Parenchymal cells of liver, kidney, or other organs did not reveal the presence of stored material. In contrast to degraded carrageenan, carrageenan administration did not give rise to stored material in reticuloendothelial cells nor in hepatocytes.”

“In a study in male and female rhesus monkeys (Macaca mulatta), the effects of κ/λ-carrageenan, (commercial product from Marine Colloids, Inc., Rockland; as Gelcarin HMR, lot n. 282400; approximate number-average molecular weight of 800 kDa; interpreted by the Panel as isolated from C. crispus with an approximate ratio of κ:λ type 70:30), have been compared with those of a degraded carrageenan (C16: commercial product from Glaxo, Paris, Batch L4006; interpreted by

the Panel as prepared from ι-carrageenan originally isolated from E. spinosum with an approximate ratio of ι:κ:λ type 100:0:0), with a number-average molecular weight of about 20 kDa (Benitz et al., 1973). Both types of carrageenan were administered in drinking-water for 7–14 week. Monkeys given 1% carrageenan (providing an intake of about 1,300 mg/kg bw per day) all gained weight and stayed in good condition. After 7 and 11 weeks, two animals were sacrificed and the remaining ones were put back on plain tap-water and allowed to recover for 11 weeks. To these animals, increasing daily doses of carrageenan ranging from 50 to 1,250 mg/kg bw per day were given for up to 12 weeks…..For the native carrageenan (κ-/λ- carrageenan), soft stools were reported with sporadic positive results for faecal occult blood with a more or less similar pattern in the control monkeys. Gross and microscopic examination of the intestinal tract revealed no changes attributable to the administration of κ-carrageenan. Of the two animals sacrificed at 7 and 14 weeks, only distal colon focal capillary hyperaemia and sometimes a slight degree of mucosal oedema was reported. All other parts of the gastro-intestinal tract were microscopically normal."

"In groups of 3–5 male and 5–7 female rats (no further information about the strain) fed 2%, 5%, 10%, 15% or 20% (equivalent to 1,800, 4,500, 9,000, 13,500 and 18,000 mg/kg bw per day) of κ/λ-carrageenan (commercial product Seakem, type 6, Marine Colloids Inc., Rockland, Main; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30) over 23–148 days via diet, apart from a decrease in weight gain in rats dosed with ≥ 10% no adverse effects were reported (Hawkins and Yaphe, 1965). No indication about weight-average molecular weight was given. As ≥ 90% of the ingested carrageenan was excreted via faeces, the authors concluded that it had no direct nutritional value.

Three groups (15 males and 15 females each) of 30 Sprague–Dawley rats of 4 weeks were exposed to 1 or 5% carrageenan (commercial product Iridaea carrageenan from Hercules Inc., Copenhagen Pectin Fabrik Ltd., Denmark; interpreted by the Panel as isolated from Iridaea ciliate = Sarcothalia crispate, mixture of κ and λ) via diet (equivalent to 900 and 4,500 mg/kg bw per day) or with cellulose at a 5% dietary level (13 males and 17 females) for 13 weeks (Abraham et al., 1985). No adverse effects on mortality, food consumption, body weight, haematology, clinical chemistry, urinalysis, faeces, or organ weights were noted in animals exposed to 1% carrageenan. No mortality was observed in animals exposed to 5% carrageenan. No indication about weight-average molecular weight was given. At this dose, one male rat showed diarrhoea in week 8 and 5/30 rats, both males and females (exact numbers not given) showed soft stools during weeks 10–13 indicating gastrointestinal disturbances. An increase in food consumption was observed in males at the highest dose (5%) compared to controls. At the highest dose (5%), in both sexes haemoglobin values were decreased compared with controls, while haematocrit levels and erythrocyte and leukocyte counts were comparable with controls. Decreased levels of Na+ and K + and increased SGPT (serum glutamic pyruvic transaminase) levels were observed in females. The faecal consistency at termination was comparable for rats of all groups and no occult blood was detected. There were also no changes in organ weights between treated and control animals. Histopathological examination revealed no changes attributable to the ingestion of carrageenan for 13 weeks. The Panel considered 4,500 mg/kgbw per day, the highest dose tested as the NOAEL in this study.

Three types of carrageenan (differing in viscosity, cation content, and ratios of κ to λ) were tested on Sprague–Dawley rats: (1) TYPE S-70:30 κ:λ ratio, high viscosity (270 cP) and high sodium (6%), (2) TYPE C-80:20 κ:λ, viscosity of 190 cps and high potassium (6%) and (3) TYPE AX-90:10 κ:λ, low viscosity (26 cP) and high calcium (6%) (Abraham et al., 1985) (for the three types, commercial products Carastay carrageenan, from Stauffer Chemical Company; sources unknown; interpreted by the Panel as an approximate ratio of ι:κ:λ type 0:70:30; 0:80:20 and 0:90:10). No indication about weight-average molecular weight was given. Eight groups of 50 animals (25 males and 25 females) received the following treatment via their diets for 13 weeks: group 1 was given ground rodent diet and served as untreated control, group 2 was given 5% cellulose and served as a control, group 3 was given 1% (equivalent to 900 mg/kg bw per day) Type S carrageenan, group 4 was given 5% (equivalent to 4,500 mg/kg bw per day) Type S carrageenan, group 5 was given

1% (equivalent to 900 mg/kg bw per day) Type C carrageenan, group 6 was given 5% (equivalent to 4,500 mg/kg bw per day) Type C carrageenan, group 7 was given 1% (about 900 mg/kg bw per day) Type AX carrageenan, and group 8 was given 5% (about 4,500 mg/kg bw per day) Type AX carrageenan. The remaining 10 males and 10 females of group 9 were killed before the start of the study for the determination of baseline haematology and blood chemistry. All parameters classically recommended in OECD 408 were measured in this study. In total, 8 out of 400 rats from groups 2, 3, 4, 7 and 8 died during the study and the cause of death (pneumonia, cage trauma, technician error) was incidental in each case and not related to treatment with carrageenan. There were no adverse effects on body weights, and food consumption was comparable within all groups. Haematological and clinical chemistry parameters gave no significant differences among the nine groups of rats and there was no increase in faecal occult blood. At necropsy, there were no gross changes caused by the treatment and both qualitative and quantitative analyses of liver tissues gave no indication for tissue residues of carrageenan (Abraham et al., 1985). The Panel considered the NOAEL of this study to be 4,500 mg/kg bw per day for all types of carrageenan tested, the highest doses tested."

"Overall, short-term and subchronic administration of carrageenan was well tolerated and induced no adverse effects in the GI tract in rats, guinea pigs, pigs and monkeys."

"Male and female mice (10 animals/group, strains not specified) were exposed for the lifetime to diets containing carrageenan (Nilson and Wagner, 1959). Carrageenan (commercial product Krim-Ko-gel, high viscosity, now designated Sea Kem, type 5; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30) used in the study (performed from 1951 to 1953) was purchased on the open market except for several lots which were supplied by the manufacturer. The first two lots analysed 76% and 75% hydrocolloid, 12% and 15% moisture, 25% and 22% mineral matter by weight, respectively. The hydrocolloid content was based on precipitating a 2% solution of the sample with 2 volumes of 99% isopropanol. No indication about the weight-average molecular weight was given. The arsenic content varied from 0.04 to 10.4 ppm (10.4 mg/kg). Carrageenan was mixed into the diet at 1%, 5%, or 15% levels (equivalent to 1,500, 7,500, 22,500 mg/kg bw per day) at the expense of equal weights of ground yellow corn and at the 25% (equivalent to 37,500 mg/kg bw per day) level for a fixed ration of ground yellow corn, wheat and oats. The animals were weighed once weekly and also food and water intake was determined once a week. No haematological and clinical parameters were measured. The mice were kept until death and at necropsy a gross examination was performed. If possible, organ sections or entire organs of each animal were preserved, sectioned and examined microscopically. No adverse effects on growth, survival, gross and histopathology were reported in any of the dosed animals.

The Panel noted that different lots of carrageenan have been used during the study, that the presence of arsenic at higher level than EU specifications in the substance has been detected. Moreover, the study has been conducted until the death of animals and there are differences in the feed composition for animals exposed to 25%. Consequently, this study cannot be used for the hazard characterisation."

"A similar study has been performed by Nilson and Wagner (1959) with groups of 10 male and female rats. The strain was not specified. Carrageenan (commercial product Krim-Ko-gel, high viscosity, now designated Sea Kem, type 5; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30) was mixed into the diet at 1%, 5% or 15% (equivalent to 500, 2,500, or 7,500 mg/kg bw per day) at the expense of equal weights of ground yellow corn, and at 25% (equivalent to 37,500 mg/kg bw per day) for a fixed ration of ground yellow corn, wheat and oats. No indication about the weight-average molecular weight was given. No adverse effects were noted except at the highest dose (25%) which exhibited lesions suggesting hepatic cirrhosis. According to the authors, these observations could indicate some dietary deficiency or insufficiency since the bulk handled was large and faeces often very loose indicating that mechanical loss of nutrient could not be discounted for this effect.

The Panel noted that different lots of carrageenan have been used during the study, and that the presence of arsenic in the test material was at higher level than EU specifications for the substance. Moreover, the study was conducted until the death of animals and there were differences in the feed composition for animals exposed to 25% carrageenan. Consequently, the Panel noted that this study was not in agreement with the current guidelines for a long-term study used for the hazard characterisation. The Panel noted that JECFA used this study in 1974 to allocate an ADI of 75 mg/kg bw per day, based on a NOAEL of 15% equal according to JECFA to 7,500 mg/kg bw per day. In 1978, the SCF endorsed this value and confirmed it until the latest evaluation in 2003.

Nutritional studies were conducted with Sprague–Dawley rats fed diets containing 4% κ/λ-carrageenan (interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30) (equivalent to 2,000 mg/kg bw per day) that had been mixed into skimmed milk at a concentration equal to that of the protein and heat sterilised under conditions routinely used in the manufacture of canned liquid milk (Tomarelli et al., 1974). No indication about the weight-average molecular weight of the tested material was given. The authors only refer to a Food and Drug Administration review (FDA, 1972) in which, to ensure against the introduction of degraded carrageenan into the food supply, have amended the existing Regulation by stipulating that the carrageenan have an average molecular weight exceeding 100 kDa, as indicated by a specified test."

"Two experiments of 6 months duration each were conducted to determine the effect of feeding relatively large amounts of processed carrageenan for prolonged periods. In the first experiment, two dietary groups were maintained: a control group fed the basal diet and a carrageenan group fed the basal diet in which equivalent amounts of the skimmed milk powder and cellulose were replaced by the processed milk-carrageenan product. In the second experiment, a third dietary group was included, a group fed the basal diet in which dextrose was substituted for the cellulose. This group served as a control to assess the influence of the relatively large amounts of indigestible carbohydrate presented by the carrageenan or cellulose. The dietary groups of the first experiment consisted of 25 males and 25 females, while in the second there were 12 males and 12 females per group. In the second experiment half of the rats of each dietary and sex group were killed at the fourth month. The rats were weighed twice weekly. At intervals, 6–8 rats from each of the groups were located in metabolism cages during 5–7 days for studying the absorption of nutrients. At necropsy, the organs were examined for gross lesions with particular attention directed to the lower gastrointestinal tract. The authors reported that, 'when fed in a simulated milk powder diet, the processed carrageenan at a dietary level of 4% had no influence (compared to glucose or cellulose) on general health, growth rate, diet energy efficiency, absorption of protein, fat, calcium, blood coagulation, utilization of protein for growth, or utilization of iron. Gross and microscopic examination of the caecum and colon revealed no abnormalities'.

The Panel considered 4% of carrageenan in the diet (equivalent to 2,000 mg/kg bw per day), which did not induce nutritional imbalance, as a NOAEL.

Several carrageenan products (commercial products from Carrageenans Hercules Inc) extracted From C. crispus (77.7% κ, 22.3% λ), G. acicularis (23.3% κ, 76.7% λ)….. were tested in rats by oral administration (Documentation provided to EFSA n. 44). No indication about the weight-average molecular weight was given. Alphacel was used as a bulk replacement control diet. Seven groups of 30 Sprague–Dawley rats (15 males, 15 females) with an age of about 4 weeks were dosed via diet with 1 or 5% (equivalent to 500 and 2,500 mg/kg bw per day) of the carrageenan or with 5% Alphacel over 40 weeks. Mortality and morbidity were recorded daily and animal weights were recorded weekly. Haematological tests included haemoglobin, haematocrit, erythrocyte count, and total and differential leukocyte count. Clinical tests consisted of blood glucose, blood urea nitrogen, serum glutamate pyruvate transaminase, Na^+, K^+, Cl^-, and examination of stools from six males and six females of each group was performed after 40 weeks. At termination, a full complement of tissue specimens was obtained from six male and six female rats dosed with 5% of the different

carrageenan and these specimens were prepared and evaluated histologically and also liver portions were processed for electron microscopy. None of the dosed rats died during the study and there was no evidence of toxicity. There were also no differences in mean body weights between dosed rats and controls receiving 5% Alphacel, and the treatment had no adverse effects on haematological and clinical chemistry parameters. The faecal consistency at study termination was comparable for rats of all groups, and occult blood was detected less frequently among rats dosed with carrageenan compared with those rats treated with Alphacel. The dosing with the different carrageenan caused no significant changes of absolute liver weights in dosed rats and the histopathological examination gave no indications for alterations in morphology or for storage of metachromatic material in either Kupffer cells or hepatocytes. The Panel considered that the NOAEL of this study was equal to 5% equivalent to 2,500 mg/kg bw per day the highest dose tested, whatever the carrageenan tested.

Carrageenan (commercial product Gelcarin HMR from Marine Colloids, Inc. Rockland, Ma, Lot No 102012; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ Type 0:70:30) was fed to two strains of weanling rats (Sprague–Dawley albino or Osborne-Mendel) at a 5% equivalent to (2,500 mg/kg bw per day) dietary level in two different basal diets (ground Wane Lab Blox [AMC] or FDA diet) for 39 weeks (Documentation provided to EFSA n. 42). Alphacel was used as a bulk replacement control diet. No indication about the weight-average molecular weight was given. Cellulose fibre was prepared at a 5% dietary level in two basal diets and fed to two strains of rats which served as controls. Rats were assigned to 8 groups of 18 males and 18 females and the rats were fed their respective diets for 39 weeks in a multiple-cross design: group l–Osborne-Mendel rats fed 5% cellulose in FDA diet, group 2–Osborne-Mendel rats fed 5% carrageenan in FDA diet, group 3–Osborne-Mendel rats fed 5% cellulose in AMC diet; group 4–Osborne-Mendel rats fed 5% carrageenan in AMC diet; group 5–Sprague–Dawley rats fed 5% cellulose in FDA diet; group 6–Sprague–Dawley rats fed 5% carrageenan in FDA diet; group 7–Sprague–Dawley rats fed 5% cellulose in AMC diet and group 8–Sprague–Dawley rats fed 5% carrageenan in AMC diet. Mortality and morbidity were recorded twice daily, and body weight, physical appearance, food and water consumption, and stool consistency (soft, firm or semifluid) were recorded at intervals. The presence of faecal occult blood was also monitored. The animals were killed and grossly examined at 30 days (3 males and 3 females per group) and 90 days (5 males and five females per group), while the surviving 160 rats were killed after 38 weeks and examined grossly. In addition, final body weights and absolute and relative liver weights were noted. Portions of the liver from all rats killed after 39 weeks were prepared for histochemical and ultrastructural determination of carrageenan storage and also a microscopic examination was undertaken. The treatment caused no mortality and all rats were in good health. Initially the body weights of Osborne-Mendel rats were lower compared with Sprague–Dawley rats, however after 39 weeks the Osborne-Mendel rats weighed more than the Sprague–Dawley strain. The dosing of carrageenan in AMC diet caused lower body weights in Sprague–Dawley males (group 8) at 4 weeks and in Osborne-Mendel females (group 4) throughout the experimental period. Soft faeces were noted in rats of all carrageenan groups. Occult blood in the faeces was sporadic in all groups (in control and treated groups) in time of appearance and quantity; occult blood was detected less frequently among rats dosed with carrageenan compared with those rats in the Alphacel group. Only Osborne-Mendel rats fed 5% carrageenan in FDA diet showed significantly increased (not further specified) relative liver weights after 39 weeks. At interim sacrifice on day 90, all rats given cellulose diet were grossly normal. Only two carrageenan fed rats (one male from group 8 and one female from group 2) had gross hepatic changes characterised as lobular flattening with a rough capsular surface. At the histopathological examination, both the 5% cellulose and the 5% carrageenan diets caused hepatocellular pericentral atrophy, hepatocellular necrosis and apoptosis. In some instances, the mitotic activity either was moderate or increased. There was a moderate degree of sinusoidal hyperaemia and varying degrees of hepatocyte vacuolisation. Bile duct proliferation was observed only in the livers of rats given carrageenan. For all of these findings, no statistics were given (Documentation provided to EFSA n. 42). The Panel considered

that the 5% in the diet equivalent to 2,500 mg/kg bw per day, the highest dose tested, was the NOAEL.

Groups of 15 male and female Sprague–Dawley rats were dosed with two extracts of carrageenan (one with molecular weight estimate of 244 kD, interpreted by the Panel as isolated from H. musciformis similar to κ; or the second one with molecular weight estimate of 252 kD, interpreted by the Panel as isolated from S. crispate (synonym: Iridaea crispata) lying between λ and κ properties) at a concentration of 0%, 1% or 5% (equivalent to 0, 500 and 2,500 mg/kg bw per day) in the diet for one year (Documentation provided to EFSA n. 43). No indication about weight-average molecular weight was given. Controls received 5% Alphacel in the diet. In rats dosed with 1% there were no changes in the stools, while female rats dosed with 5% carrageenan from S. crispate (I. crispate) and males dosed either carrageenan at the 5% concentration had loose stools. Blood was observed sporadically in the stools, but the frequency was lower than in the controls. At the end of the study, body weights of the carrageenan-treated groups were decreased by 20% or 25% in the 1% and 5% carrageenan groups, respectively. In the group dosed with 1% carrageenan from H. musciformis the absolute and relative liver weights of males were comparable to the controls, while the absolute and relative liver weights of females were decreased when compared to the controls (absolute: 9.0 vs 15.6 g; relative 3.0 vs 4.0 g). In the group dosed with 5% carrageenan from H. musciformis, the absolute liver weights of males and females were decreased compared to the controls (males: 14.9 vs 18.7 g; females 8.4 vs 15.6 g) and the relative liver weight of females was decreased compared to the controls (2.9 vs. 4.0 g). In the group dosed with 1% carrageenan from S. crispate (I. crispate) the absolute liver weight of males was comparable to the controls but the relative weight was increased (3.1 vs. 2.7 g). For females, both the absolute and relative liver weights were decreased compared to the controls (absolute: 9.8 vs 15.6 g; relative 3.2 vs 4.0 for the controls). In the group dosed with 5% carrageenan from S. crispate (I. crispata) the absolute liver weights of the females were lower than in the controls (8.2 vs 15.6 g). For this group the relative liver weights of the males were higher than the controls (3.0 vs 2.7 g) and the relative liver weight of the females was lower compared to the controls (2.9 vs 4.0 g). Some incidental effects were observed at macroscopical and histopathological examination of the liver. The dosing gave no indication for storage of carrageenan-like material in the liver cells of any of the treated rats, and no fibrillar material was seen by electron microscopy. Except for gross changes in the livers, no other adverse effects were observed. The Panel considered that the LOAEL in this study was 1% in the diet (equivalent to 500 mg/kg bw per day) for both carrageenan preparations."

As taken from EFSA, 2018.

Type of Test	Route of Exposure or Administration	Species/Test System	Dose Data	Toxic Effects	Reference
TDLo - Lowest published toxic dose	Oral	Rodent - rat	13687.5 gm/kg/2Y (continuous)	Liver - hepatitis, fibrous (cirrhosis, post-necrotic scarring)	CRTXB2 CRC Critical Reviews in Toxicology. (CRC Press, Inc., 2000 Corporate Blvd., NW, Boca Raton, FL 33431) V.1- 1971- Volume(issue)/page/year : 32,413,2002
TDLo - Lowest published toxic dose	Oral	Rodent - rat	1368.75 gm/kg/1Y (continuous)	Nutritional and Gross Metabolic - weight loss or decreased	CRTXB2 CRC Critical Reviews in Toxicology. (CRC Press, Inc., 2000 Corporate Blvd., NW, Boca Raton, FL 33431) V.1- 1971-

				weight gain	Volume(issue)/page/year : 32,413,2002
TDLo - Lowest published toxic dose	Oral	Primate - monkey	1368.75 gm/kg/7Y (intermittent)	Gastrointestinal - hypermotility, diarrhea	CRTXB2 CRC Critical Reviews in Toxicology. (CRC Press, Inc., 2000 Corporate Blvd., NW, Boca Raton, FL 33431) V.1- 1971- Volume(issue)/page/year : 32,413,2002

As taken from RTECS, 2018.

POD Method	POD Value	POD Owner
HNEL	Not Calculated	PAFA

Lowest-observed effect

Owner	Type	Value	Sites	Effects
PAFA	LOAEL	1656.0 mg/kg bw/day	• CLINICAL SIGNS	• CLINICAL SIGNS

No-observed effect: PAFA: HNEL: Not established

Adjustment factors: >Critical study: Target Organ Toxicity > Subchronic Toxicity (Rat, Oral exposure) for 90 day

As taken from the COSMOS database, available at https://ng.cosmosdb.eu

In 2018, the EFSA ANS Panel delivered an opinion on the re-evaluation of carrageenan (E 407) and PES (E 407a) as food additives. The ANS Panel concluded that'...the ADME database was sufficientto conclude that carrageenan was not absorbed intact; in a sub-chronic toxicity study performed withcarrageenan almost complying with the EU specification for E 407 in rats, the no-observed-adverse-effect level (NOAEL) was 3,400–3,900 mg/kg bw per day, the highest dose tested; no adverse effectshave been detected in chronic toxicity studies with carrageenan in rats up to 7,500 mg/kg bw per day,the highest dose tested.

As taken from EFSA, 2022

5.3. Reproduction toxicity

No data available to us at this time.

5.4. Mutagenicity

"Food-grade carrageenan (commercial product purchased on market place as food grade and analysed by the South Korean advanced Food Research Institute according to Korea Food Additive Code; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30, number- or weight-average molecular weight not specified) was tested in a bacterial reverse mutation assay. At the tested concentrations of 156.3–5,000 µg/plate, both with and without metabolic activation, there was no indication for mutagenicity in the tester strains of S.Typhimurium TA1535,TA1537, TA98, TA100 and in Escherichia coli WP2uvrA (Chung et al., 2009). The Panel noted that the study meets the requirements of the relevant OECD TG Guideline no. 471."

"Food-grade carrageenan (commercial product purchased on market place as food grade and analysed by the South Korean Food Research Institute according to Korea Food Additive Code; the origin cannot be interpreted by the Panel, number- or weight-average molecular weight not specified) was assessed for the induction of chromosomal aberrations in Chinese hamster lung

fibroblast (CHL), and for induction of micronuclei and DNA breakage by means of the alkaline comet assay in the L5178Y mouse lymphoma cells (Chung et al., 2009). For the induction of chromosomal aberrations, the assay consisted of short-term (6 h) both in the absence and presence of S9 metabolic activation and continuous (24 h) treatments in the absence of S9 metabolic activation at dose levels of 150, 300 and 600 µg/mL. Approximately 22 h after the beginning of the treatment, colcemid was added to each culture at a final concentration of 0.25 µg/mL. 200 metaphases (100 metaphases from each duplicate culture) were selected and analysed for each treatment group and the results were expressed as mean aberrant metaphases excluding gaps per 100 metaphases. At the tested concentrations of 150–600 µg/mL, there was no indication of a clastogenic effect. The Panel noted that the study is compliant with the OECD TG Guideline no 473.

For the induction of micronuclei, L5178Y mouse lymphoma cells were treated with carrageenan at concentrations of 0, 50, 100 or 200 µg/mL for 3 h, both, with and without S9 mix and harvested after a 21-h recovery period, or treated at concentrations of 0, 50, 100 or 200 µg/mL for 24 h without S9 mix and harvested immediately. No duplicate cultures were used and micronuclei were scored in 2,000 cells per culture. Slight but not significant increases in micronucleated cells compared to the concurrent vehicle control were observed at all dose levels and treatment performed. The observed increases were considered by the authors to be of no biological relevance (Chung et al., 2009). The Panel agrees with this conclusion and noted that the study is compliant with the OECD TG no. 487, with the exception that the highest dose level selected was not sufficiently high due to the absence of cytotoxicity and did not show any precipitation of test compound in culture medium as observed in the chromosomal aberration assay. In addition, no historical control range values were reported.

For the comet assay, L5178Y mouse lymphoma cells were similarly treated with carrageenan at concentrations of 0, 50, 100 or 200 µg/mL for 3 h, both, with and without S9 metabolic activation and harvested after a 21-h recovery period, or treated at concentrations of 0, 50, 100 or 200 µg/mL for 24 h without S9 metabolic activation and harvested immediately. Duplicate cultures were used and comets were examined from 100 randomly selected nuclei (50 nuclei per culture) using a fluorescence microscope. Images of selected nuclei were analysed using image-analysis software and tail intensity (% of tail DNA) and Olive tail moment were used to measure the damage of DNA damage. Slight but statistically significant increases in % tail DNA and Olive tail moment compared to the concurrent vehicle control were sporadically found. The increases were not dose-related and the observed values were similar to those commonly observed in the untreated controls for this cell line (Chung et al.,2009). However, the Panel noted some shortcomings which include the absence of historical control range values, a limited number of cells scored, a limitation in the selection of the highest dose level which did not cause adequate cytotoxicity, and no precipitation was observed in culture medium as it was in the chromosome aberration assay.

The Panel considered that carrageenan proved to be negative in all end-points tested in this study (Chung et al., 2009)."

"Overall, the Panel concluded that carrageenan….. did not raise a concern with respect to genotoxicity…..the food additives E 407 and E 407a, even if they contain some low molecular weight carrageenan, were not of genotoxic concern."

As taken from EFSA, 2018.

Ingredient/Similar Chemical	Strain/cell type	Assay	Dose	Results
Bacterial Assays				
Carrageenan (natural grade	Salmonella typhimurium strain	Ames test	Concentrations up to 100 mg/ml (PNG)	Not genotoxic

[PNG]) or refined Carrageenan	TA100		and up to 25 mg/ml (refined) without metabolic activation	
kappa/lambda-Carrageenan (from C. crispus	Salmonella typhimurium strains TA1535, TA1537, and TA1538. Saccharomyces cerevisiae strain D4.	Ames test	Test concentrations not stated	Not genotoxic
PNG or Refined Carrageenan	Mice (strain not stated). Salmonella typhimurium strain His G 46	Host-mediated assay	Mice received PNG at oral doses up to 2,500 mg/kg body weight or refined carrageenan at a dose of 700 mg/kg body weight. Bacterial strain tested without metabolic activation	Mutation frequency in injected indicator organism not affected by dosing with carrageenan. Neither PNG nor refined carrageenan was genotoxic.
PNG or Refined Carrageenan	Bacillus subtilis	Rec assay for DNA - damaging potential	PNG and refined carrageenan tested at concentrations up to 100 mg/ml and 28 mg/ml, respectively	Neither PNG nor refined carrageenan was genotoxic
Mammalian Assays				
PNG or Refined Carrageenan	Bone marrow cells from Swiss mice	Micronucleus test	Mice received PNG at doses up to 2,500 mg/kg body weight or refined carrageenan at a dose of 700 mg/kg body weight	Neither PNG nor refined carrageenan was genotoxic

As taken from CIR, 2015.

Type of Test	Route of Exposure or Administration	Species/Test System	Dose Data	Reference
DNA inhibition		Rodent - mouse Cells - not otherwise specified	2 gm/L	CALEDQ Cancer Letters (Shannon, Ireland). (Elsevier Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1- 1975- Volume(issue)/page/year: 148,115,2000

As taken from RTECS, 2018.

5.5. Cytotoxicity

“Another study assessed whether binding of carrageenan to TLR4 was specific or due to the mechanical coating of the membrane as a result of the large molecular weight of carrageenan and

of the conditions used in vitro (Documentation provided to EFSA n. 47). Human embryonic kidney cells transfected with a TLR4 reporter system were employed to determine the binding, using alkaline phosphatase (SEAP) as the reporter molecule. Cells were exposed for 24 h to various types of carrageenan, all compliant with the JECFA specifications: food-blend carrageenan from a manufacturer….. and a commercial mixture of κ- and ι-carrageenan, at concentrations of 0, 0.1, 1, 10, 50, 100, 500, 1,000 or 5,000 ng/mL'. Positive (LPS) and negative (clarified locust bean gum (CLBG) and sodium alginate (SA)) controls were included. Cell viability, as measured by the levels of cellular adenosine triphosphate and released lactate dehydrogenase released, was unaffected by any of the carrageenan or by CLBG, SA or LPS at any exposure concentration tested. Overall, no measurable changes in cell viability were observed under these test conditions, and the three types of carrageenan tested were not TLR4 agonists or antagonists."

As taken from EFSA, 2018.

Carrageenan is a highly potent anti-human papillomavirus (HPV) agent with the potential for formulation as a mouthwash against oral HPV infection. However, its toxic effect on tissues of the oral cavity is currently unknown. This study aims to evaluate the safety of carrageenan on human cells and tissues of the oral cavity. Human salivary gland cells and reconstructed human oral epithelium (RHOE) were used for this in vitro study. The cells were subjected to 0.005-100 µg/mL of carrageenan for 4, 12, and 24 h in quadruplicate. RHOE were exposed to 100 µg/mL of carrageenan for 24 h in triplicate and stained with hematoxylin/eosin for histological analyses. All experiments had saline and 1% sodium dodecyl sulphate (SDS) as negative and positive controls, respectively. Carrageenan tissue toxicity was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to quantify cell viability. Tissue toxicity was further evaluated histologically by an oral pathologist to assess morphological changes. Our data showed that carrageenan did not significantly decrease cell and tissue viability when compared to the positive control. The histological evaluation of the RHOE also showed no loss of viability of the carrageenan-treated sample compared to untreated tissue. In contrast, 1% SDS-treated RHOE showed extensive tissue destruction. Our experiments suggest that carrageenan is safe for use in the oral cavity.

As taken from Alli BY et al. 2022

5.6. Carcinogenicity

"Groups of 30 male and 30 female MRC outbred rats were dosed lifetime with 0%, 0.5%, 2.5% or 5% of carrageenan (commercial product Gelcarin HMR from Marine Colloids, Inc., Rockland, ME, Lot. No 102012; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30), in the diet (Rustia et al., 1980). Carrageenan (Gelcarin) was reported as having a molecular weight of approximately 800 kDa. No indication about weight-average molecular weight was given. The average daily carrageenan intake was equal to 0, 360, 1,998 and 4,022 mg/kg bw. As untreated controls, 100 females and 100 males were used. The animals were inspected routinely two times a day and food consumption was recorded twice a week for a 2-day period. Animals were allowed to die spontaneously or killed when moribund. Complete autopsies were performed on all experimental and control animals, except for a few that were cannibalised or showed advanced post-mortem deterioration. Histological specimens were taken from all visceral organs and from all tumours and pathologically altered tissues. All segments of the gastrointestinal tract including the oesophagus, glandular steroids and forestomach, small intestine/duodenum, jejunum and ileum, cecum, colon and rectum were examined for lesions. The other tissues were fixed, processed and stained routinely with haematoxylin and eosin. The data including tumour and death rates were statistically analysed. The animals tolerated the carrageenan well at all dose levels and exhibited no clinical signs of excessive toxicity (e.g. significant body weight reduction). Animals occasionally developed a soft stool consistency, particularly in the initial experimental stages, and also diarrhoea was noted in some animals. No ulcerative alterations or neoplastic lesions of the intestinal tract were observed and the number of tumour-bearing animals, tumours

per tumour-bearing animal and the overall incidence (i.e. number of tumours in test and control animals) and average latency periods did not differ significantly among test and control groups). According to the Panel, the results of this study, demonstrated no carcinogenic effects of carrageenan in rat. The Panel considered that the NOAEL of this study was 4,022 mg/kg bw per day, the highest dose tested."

"In a study with Syrian golden hamsters, groups of 30 male and 30 female hamsters were dosed livelong with 0%, 0.5%, 2.5% or 5% of carrageenan (commercial product Gelcarin HMR from Marine Colloids, Inc., Rockland, ME, Lot. No 102012; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30) (Rustia et al., 1980). Carrageenan (Gelcarin) was reported as having a molecular weight of approximately 800 kDa. No indication about weight-average molecular weight was given. The average daily carrageenan intake was equal to 0, 370, 2,162 or 3,719 mg/kg bw per day. As untreated controls, 100 females and 100 males were used. The animals were inspected routinely two times a day and food consumption was recorded twice a week for a 2-day period. Animals were allowed to die spontaneously or killed when moribund. Complete autopsies were performed on all experimental and control animals, except for a few that were cannibalised or showed advanced post-mortem deterioration. Histological specimens were taken from all visceral organs and from all tumours and pathologically altered tissues. All segments of the gastrointestinal tract including the oesophagus, glandular steroids and forestomach, small intestine/duodenum, jejunum and ileum, cecum, colon and rectum were examined for lesions. The other tissues were fixed, processed and stained routinely with haematoxylin and eosin. The data including tumour and death rates were statistically analysed. The animals tolerated the carrageenan well at all dose levels and exhibited no clinical signs of excessive toxicity (e.g. significant body weight reduction). Animals given the highest dose level of carrageenan had soft stools during the initial weeks of the experiment. No ulcerative alterations or neoplastic lesions of the intestinal tract were observed and the number of tumour-bearing animals, tumours per tumour-bearing animal and the overall incidence (i.e. number of tumours in test and control animals) and average latency periods did not differ significantly among test and control groups). From the results of this study, carrageenan demonstrated no carcinogenic effects in Syrian golden hamsters. The Panel considered that the NOAEL of this study was equal to 3,719 mg/kg bw per day the highest dose tested."

"A long–term toxicity study (7.5 years) has been performed on 40 rhesus monkeys (19 males and 21 females with initial weights of 2–8.22 kg) which were divided into 4 groups with 10 animals each and dosed with 0, 50, 200, 500 mg/kg bw per day κ/λ-carrageenan (commercial product Gelcarin HMR from Marine Colloids, Rockland, Maine; interpreted by the Panel as isolated from C. crispus with an approximate ratio of ι:κ:λ type 0:70:30) by gavage daily on six days a week for the first 5 years (after about one year 10/19 males became aggressive and were dosed via diet) (Documentation provided to EFSA n. 34). No indication about weight-average molecular weight was given. Thereafter, for the remaining 2.5 years, carrageenan was incorporated into diet and given to monkeys. Observations on appearance and behaviour were done on 6–7 days/week, while body weights were recorded weekly for the first month, and monthly thereafter and before termination. Clinical chemistry was done twice prior to start and at 2–12 months, in years 4–7 and prior to termination. A hepatic biopsy was performed on selected monkeys after 9 months and in years 2, 3.75, 5 and 6 of the study. Necropsy and gross examination was done in 6 of 10 monkeys which died or were killed (2–3 animals within all groups) for humane reasons during the study and at termination. The organ weights of liver, kidneys, heart, spleen, ovaries, testes, brain, adrenals and pituitary were determined and a microscopic examination of all major relevant organs was done. Histopathological examination (staining with toluidine blue) and electron microscopy was done in portions of the liver, large and small intestine, caecum and lymph nodes from each monkey. No statistical differences in mean survival times were detected in any group as compared to controls and there were also no gross or microscopic changes in the examined tissues. During years 5–7.5, in females dosed with 50 mg/kg bw per day a significant decrease in body weights was seen. Loose stools, chronic intestinal disorders, poor appetite and emaciation were observed

in an apparently random distribution. Stool consistency was decreased in a dose-related manner over 7.5 years (the incidence of soft stools as a frequency of total observations was 9.1%, 18.7%, 20.3% and 24.4% in controls, low, mid- and high-dose groups) while positive faecal occult blood findings were increased in a similar fashion; the values obtained ranged from 16.8% in controls to 25.4% in the high-dose group. Faeces of monkeys given carrageenan were found to contain degraded carrageenan. Therefore, the Panel noted that it cannot be excluded that the presence of occult blood in animals is treatment-related. There were no changes in haematology, clinical chemistry values, organ weights, gross pathology, microscopic and ultrastructural morphology. There was also no evidence for the uptake of fibrillar, metachromic, carrageenan-like material in the cells of the reticular-endothelial system in the liver, lymph nodes, caecum, colon or anorectal junction. Three neoplastic changes were noted at termination: one perianal tumour in one control female, a cervical polyp in one female at 50 mg/kg bw per day and a fibroma of the thigh in one male at 200 mg/kg bw per day).

The Panel noted that the chronic toxicity studies were almost exclusively conducted with κ/λ-carrageenan and that no or no adequate description of the molecular weight distribution of the tested carrageenan preparations was given. There was no indication of carcinogenicity related to the treatment."

"Several initiation–promotion studies with equivocal data were identified in the scientific literature by the Panel. Overall, the Panel considered that the results of the initiation–promotion studies did not demonstrate consistent modulating effects of carrageenan on the initiation and/or promotion phase of colon carcinogenesis induced in rats by well-known carcinogenic compounds such as azoxymethane (AOM) (Watanabe et al., 1978; Corpet et al., 1997; Tache et al., 2000) and dimethyhydrazine (DMH) (Arakawa et al., 1986; Hagiwara et al., 2001). Watanabe et al. (1978) and Arakawa et al. (1986) used doses up to 15% of the diet and were in excess of those accepted as maximum (5%) for a chronic bioassay (OECD, 1998). Aberrant crypt foci were used as putative preneoplastic (surrogate) markers for the development of colon cancer in the studies of Corpet et al. (1997) and Tache et al. (2000). They found a significant decrease in the number of aberrant crypt foci. Furthermore, no plausible mechanism of action has been proposed that could explain the results observed.

Overall, the Panel considered that there was no concern with respect to the carcinogenicity of carrageenan in several chronic toxicity studies in rats performed with carrageenan (mostly κ/λ-type, molecular weight distribution not adequately described). From the available rats studies, NOAELs up to 7,500 mg/kg bw per day, the highest dose tested, were identified (Nilson and Wagner, 1959). However, in another study in rats given carrageenan preparations with a molecular weight of 244 kDa and 252 kDa, a LOAEL of 1% in the diet (equivalent to 500 mg/kg bw per day) was identified by the Panel (Documentation provided to EFSA n. 43). The Panel noted that the characterisation of the test material in all the chronic studies was limited."

As taken from EFSA, 2018.

"Carrageenan: 0.1, 5, 15, and 25% carrageenan. Groups of 5 male and 5 female mice of two strains. Feeding in the diet for lifespan. Additional group fed control diet. Non-carcinogenic.

Carrageenan: 1, 5, 15, and 25% carrageenan. Groups of 5 male and 5 female mice of two strains. Feeding in diet for up to 24 months. Additional group fed control diet. Hepatic sclerosis at 25% concentration. Non-carcinogenic."

"Groups of female Fischer 344 rats. Co-carcinogenicity of carrageenan in presence of azoxymethane (AOM) or N- nitrosomethylurea (NMU) evaluated. Treatment groups: control diet (15 rats); 15% carrageenan in control diet (15 rats); 15% carrageenan in control diet + 10 weekly s.c. injections of 8 mg/kg bw (AOM) (30 rats); 2 mg NMU (intrarectal instillations) twice weekly for 3 weeks (30 rats); AOM s.c. alone (30 rats), and NMU i.r. alone (30 rats). Animals killed 40 weeks after the initial injection of AOM or 30 weeks after the initial injection of NMU. Carrageenan

enhanced the incidence of colon tumors in AOM- and NMU-treated rats ($p < 0.01$): AOM + carrageenan (26/26, 100%) versus AOM alone (17/30, 57%); NMU + carrageenan (29/29, 100%) versus NMU alone (20/29, 69%); control diet (0/15); and 15% carrageenan in control diet (1/15, 7%)."

"An epidemiology study was performed to examine the hypothesis that the increasing incidence of mammary carcinoma in the United States in the twentieth century may be related to the consumption of carrageenan and possibly other water-soluble polymers. A time-trend analysis using age-adjusted incidence data and consumption data from established sources was used to test this hypothesis. Statistical analysis, using Pearson and Spearman correlation coefficients, was performed to identify associations between water-soluble polymer consumption and cancer incidence. Lag periods of 10, 15, 20, 25, 30, and 35 years were introduced to consider a latent effect between intake and the occurrence of breast cancer.

At least 4 values for consumption and corresponding incidence were required for inclusion in the correlation analysis. Consumption data on the polysaccharide gums studied were reported as pounds/person/year. These water-soluble polymer utilization data, obtained from several libraries throughout the United States, were predominantly from published data compiled as research for the food industry. For carrageenan, 80% of total consumption was identified as food consumption, and the remainder was attributed to products such as toothpaste, deodorants, room deodorizers, etc.…

The following positive correlations between gum consumption and the incidence of mammary carcinoma were found. For carrageenan, positive correlations (statistically significant) were found at 25 years ($r = 0.88$; $P = 0.048$) and 30 years ($r = 0.96$; $P = 0.042$). The Spearman correlation coefficient for carrageenan at 30 years was also statistically significant ($r = 1.0$; $P < 0.0001$)."

As taken from CIR, 2015.

Type of Test	Route of Exposure or Administration	Species/Test System	Dose Data	Toxic Effects	Reference
ICLo - Inhibitor Concentration Low	In vitro	Human - colon tumor	1 mg/L/1H	In Vitro Toxicity Studies - other assays	TOLED5 Toxicology Letters. (Elsevier Science Pub. B.V., POB 211, 1000 AE Amsterdam, Netherlands) V.1- 1977- Volume(issue)/page/year: 211,289,2012
ICLo - Inhibitor Concentration Low	In vitro	Human - colon tumor	1 umol/L/120H	In Vitro Toxicity Studies - apoptosis in vitro	TOLED5 Toxicology Letters. (Elsevier Science Pub. B.V., POB 211, 1000 AE Amsterdam, Netherlands) V.1- 1977- Volume(issue)/page/year: 231,1,2014
TDLo - Lowest published toxic dose	Oral	Rodent - rat	2100 gm/kg/40W (continuous)	Tumorigenic - equivocal tumorigenic agent by RTECS criteria Gastrointestin	CNREA8 Cancer Research. (Public Ledger Building, Suit 816, 6th & Chestnut Sts., Philadelphia, PA 19106) V.1- 1941- Volume(issue)/page/ye

				al - colon tumors	ar: 38,4427,1978
TDLo - Lowest published toxic dose	Subcutaneous	Rodent - rat	525 mg/kg/21W (intermittent)	Tumorigenic - neoplastic by RTECS criteria Tumorigenic - tumors at site of application	13BYAH "Morphological Precursors of Cancer, Proceedings of an International Conference, Perugia, Italy, 1961," Severi, L., ed., Perugia, Univ. of Perugia, 1962 Volume(issue)/page/year: -,83,1962
TDLo - Lowest published toxic dose	Parenteral	Rodent - rat	430 mg/kg	Tumorigenic - equivocal tumorigenic agent by RTECS criteria Skin and Appendages - tumors	BJCAAI British Journal of Cancer. (Macmillan Press Ltd., Houndmills, Basingstoke, Hants. RG21 2XS, UK) V.1- 1947- Volume(issue)/page/year: 15,607,1961
TD - Toxic dose (other than lowest)	Parenteral	Rodent - rat	320 mg/kg	Tumorigenic - equivocal tumorigenic agent by RTECS criteria Skin and Appendages - tumors	OYYAA2 Oyo Yakuri. Pharmacometrics. (Oyo Yakuri Kenkyukai, CPO Box 180, Sendai 980-91, Japan) V.1- 1967- Volume(issue)/page/year: 32,711,1986

As taken from RTECS, 2018.

5.7. Irritation/immunotoxicity

“Weiner et al. (2015) evaluated the potential effects of carrageenan after 28 days of administration of piglet-adapted formula to Domestic Yorkshire Crossbred Swine (6 males, 6 females per group) from lactation day 3 for 28 consecutive days on several endpoints. The endpoints evaluated included emphasis on the GI tract and the immune system including immunophenotyping, circulating cytokines and immunohistochemistry of the GI tract. Animals were exposed to 0, 300, 1,000 and 2,250 mg/kg diet equal to 0, 52, 193, and 430 mg/kg bw per day and 0, 56, 203, and 448 mg/kg bw per day, for male and female, respectively. The test material sample of κ/λ-carrageenan (FMC Lot. 90303011) (ratio κ/λ unknown) was fully characterised for, molecular weight, percentage of the molecular weight below 50 kDa, known as the low molecular weight tail (LMT) as per European requirements, and met internationally recognised carrageenan food additive specifications. The weight-average molecular weight of the test material sample was 664–732 kDa with a LMT of 0.3–3.9% and a viscosity of 80 cP. Some animals show minor findings, such as faecal changes but clinical pathology parameters, including haematology, clinical chemistry and coagulation were unaffected by the treatment. The results of the immunological evaluations did not reveal any treatment-related changes at the concentrations tested as compared to the vehicle control (swine adapted infant formula).”

“The α-1,3-galactosidic linkage, which is present in the three types of carrageenan is specifically recognised by human IgG and IgM anti-galactose natural antibodies. Binding of these antibodies to this saccharide epitope may lead to an immune reaction and, for instance, they were involved in acute rejection by humans of pig xenografts (Galili et al., 1985; Thibaudeau et al., 1996; Klein et al., 1998). These antibodies developed with age in human; they are not present in the neonates but develop within a few months, as soon as the newborn’s gastrointestinal tract becomes colonised by microorganisms.

The Panel noted that an ELISA was available for the detection/quantification of carrageenan in various foods (Haines and Patel, 1997, 2005), which indicated that carrageenan could elicit the production of and react with, specific antibodies thus revealing the presence of immunogenic sites in carrageenan.”

“Vojdani and Vojdani (2015) reported that 27%, and 17% of the sera from 288 healthy, asymptomatic volunteers contained specific IgG and IgE antibodies to carrageenan, respectively. Fixation of IgE but not of IgG antibodies was largely inhibited by several common food antigens, indicating that these IgE may be formed in response to cross-reacting antigens present in common foods.

The Panel noted that despite the presence in human serum of antibodies capable to recognize saccharidic epitopes on the carrageenan molecules, no case reports of significant allergic and/or anaphylactic reactions after ingestion of foods containing carrageenan were identified in the available literature.”

“In a series of studies with various cell models (RAW 264.7 and 23ScCr mouse macrophage cell lines, NCM 460 human colonic epithelial cell line, primary cultures of human colonic cells), Bhattacharyya et al. (2008b, 2010) studied the mechanisms of carrageenan-induced intestinal inflammation. Toll-like receptor (TLR) and the protein B-cell lymphoma/leukaemia 10 (BCL10) were identified as important components of the cell membrane for binding of carrageenan to the cell membrane. The authors reported that in their studies, binding of carrageenan (purity and composition not reported) to the receptor resulted in stimulation of NF-κB, and activation of interleukin-8 (IL-8) production and of a reactive oxygen species (ROS)-mediated pathway.

Choi et al. (2012) evaluated the effects of carrageenan on proinflammatory transcription factor NF-κB (subfamily RelA/p65) and early growth response gene 1 product (EGR-1) in relation to human intestinal epithelial barrier integrity. Human colonic cancer cell lines HCT-8, HT-29 and Caco-2 were exposed to 0 or 1µg/mL of carrageenan (type not specified) for up to 24 h. NF-κB activation (but not EGR-1 activation) was involved in the induction of proinflammatory cytokine IL-8. According to the authors, the results showed that both NF-κB and EGR-1 play a role in maintaining the epithelial barrier integrity in response to carrageenan.”

“Sherry et al. (1993; letter to the journal editor) assessed whether there was a correlation between the frequency of symptomatic upper respiratory tract infections and the type of formula during the first 6 months of life in full term-infants. No indication about weight-average molecular weight was given. In this study, 1,269 infants given liquid formula containing carrageenan at 300 mg/L (0.03%) were compared with 149 infants given powder-based formula without carrageenan. A slightly higher proportion of infants (53.4%) given liquid infant formula containing carrageenan were illness-free during the first 6 months of life compared to those receiving powder-based formula without carrageenan (47.6%). A slight difference in the number of months in which at least one upper respiratory tract infection occurred was also found in favour of the infants receiving liquid formula (0.9 month) compared to powdered formula (1 month). However, the differences between the two groups were no statistically significant. Based on the results, the authors suggested that the carrageenan-containing liquid infant formula was not immunosuppressive. The Panel considered that the details of the study reported in the letter to the journal were too limited to draw a reliable conclusion.”

As taken from EFSA, 2018.

Type of Test	Route of Exposure or Administration	Species/Test System	Dose Data	Toxic Effects	Reference
ICLo - Inhibitor Concentration Low	In vitro	Human - intestinal	1 mg/L/12 H	Biochemical - Metabolism (Intermediary) - effect on inflammation or mediation of inflammation	TOLED5 Toxicology Letters. (Elsevier Science Pub. B.V., POB 211, 1000 AE Amsterdam, Netherlands) V.1- 1977- Volume(issue)/page/year : 211,289,2012

As taken from RTECS, 2018.

“The present study characterized carrageenan inflammatory nociception in the 7-day-old domestic chick. The time course effects of foot withdrawal latency to a thermal stimulus and edema were examined over a 6-h period following an intraplantar carrageenan (0.0-1.0%) injection. Carrageenan-induced hyperalgesia and edema had a similar course of action, enduring for approximately 6 h, with a peak effect at approximately 2 h post carrageenan injection. Carrageenan inflammation was produced in a robust concentration dependent manner. Carrageenan hyperalgesia was induced at all concentrations tested and no carrageenan concentration effects were discerned. In a subsequent series of experiments we challenged the carrageenan inflammation model with systemic administration of the opioid agonist morphine, the nonsteroidal anti-inflammatory drug naproxen or the steroidal antiinflammatory drug dexamethasone. Morphine produced a dose dependent attenuation of carrageenan hyperalgesia but had no effect upon carrageenan inflammation. Naproxen produced a moderate attenuation of carrageenan inflammation and hyperalgesia. Dexamethasone dramatically attenuated both carrageenan hyperalgesia and inflammation. Collectively, these experiments characterize the chick carrageenan response and demonstrate the potential of the chick carrageenan inflammation model as a less expensive adjunct model of inflammatory nociception.” As taken from Roach JT and Sufka KJ. 2003. Brain Res. 994(2), 216-225. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/14642647

5.8. All other relevant types of toxicity

“The Panel further noted that according to Weiner (2014, 2016), the vehicle employed to administer carrageenan was critical for understanding the toxicological effects and their interpretation because the conformation of the carrageenan molecule changes depending on the vehicle. Most animal studies administered carrageenan in diet or in drinking water and some studies were conducted by oral gavage in water. When carrageenan was dissolved in water at dilute concentration (50.1%) with no added potassium or calcium ions, dietary solids or protein, the polymer independent of type exists in a disorganised random conformation (Blakemore and Harpel, 2010). Under these conditions, which are not representative of the use of the food additive E 407, all the carrageenan molecules are available for maximum interaction with the gastrointestinal (GI) tract cells.”

“Another masked, randomised, parallel growth and tolerance study was conducted on healthy infants (aged 0–9 days at the time of enrolment) during the first four months of life (Borschel et al., 2002). A total of 195 infants were given, from enrolment until 112 days of age, extensively hydrolysed casein–based formulas in two different forms: powdered (PWD) that did not contain carrageenan (95 infants), and liquid ready-to-feed (RTF) containing carrageenan (100 infants). No indication about the weight-average molecular weight was given. One hundred and thirty-seven infants completed the study, whereas 58 exited the study in early stages. Among these, 21 subjects from the RTF group and 16 from the PWD group left the study due to intolerance symptoms. Intake,

stool patterns and anthropometric measurements (e.g. weight, length and head circumference) were monitored at entry and on days 14 (only anthropometric parameters), 28, 56, 84 and 112 of the study. Overall, no statistically significant differences between the two groups were reported in weight gain, length, head circumference or tolerance to the formulas."

As taken from EFSA, 2018.

"Carrageenan (CGN) is a common food additive that has been widely used for decades as a gelling, thickening and stabilizing agent. Carrageenan has been proven safe for human consumption; however, there has been significant confusion in the literature between CGN and the products of intentional acid-hydrolysis of CGN, which are degraded CGN (d-CGN) and poligeenan (PGN). In part, this confusion was due to the nomenclature used in early studies on CGN, where poligeenan was referred to as "degraded carrageenan" (d-CGN) and "degraded carrageenan" was simply referred to as carrageenan. Although this nomenclature has been corrected, confusion still exists resulting in misinterpretation of data and the subsequent dissemination of incorrect information regarding the safe dietary use of CGN. The lack of understanding of the molecular weight distribution of CGN has further exacerbated the issue. The significant differences in chemistry, manufacture, and protein reactivity of CGN versus d-CGN and PGN are reviewed, in addition to the in vivo toxicological profiles of CGN, d-CGN, and PGN. As CGN cannot be hydrolyzed to PGN in vivo, concerns over the use of CGN as a food additive are unfounded, particularly since current studies support the lack of oncogenic and tumorigenic activity of CGN in humans." As taken from McKim JM et al. 2019. Crit. Rev. Food Sci. Nutr. 59(19), 3054-3054. PubMed, 2020 available at: https://pubmed.ncbi.nlm.nih.gov/29902080/

Type of Test	Route of Exposure or Administration	Species/Test System	Dose Data	Toxic Effects	Reference
ICLo - Inhibitor Concentration Low	In vitro	Human - intestinal	1 mg/L/1H	In Vitro Toxicity Studies - other assays	TOLED5 Toxicology Letters. (Elsevier Science Pub. B.V., POB 211, 1000 AE Amsterdam, Netherlands) V.1- 1977- Volume(issue)/page/year: 211,289,2012

As taken from RTECS, 2018.

"Food additives, often used to guarantee the texture, shelf-life, taste, and appearance of processed foods, have gained widespread attention due to their increased link to the growing incidence of chronic diseases. As one of the most common additives, carrageenans have been used in human diets for hundreds of years. While classified as generally recognized as safe (GRAS) for human consumption, numerous studies since the 1980s have suggested that carrageenans, particularly those with random coil conformations, may have adverse effects on gastrointestinal health, including aggravating intestinal inflammation. While these studies have provided some evidence of adverse effects, the topic is still controversial. Some have suggested that the negative consequence of the consumption of carrageenans may be structure dependent. Furthermore, pre-existing conditions may predispose individuals to varied outcomes of carrageenan intake. In this review, structure-function relationships of various carrageenans in the context of food safety are discussed. We reviewed the molecular mechanisms by which carrageenans exert their biological effects. We summarized the findings associated with carrageenan intake in animal models and clinical trials. Moreover, we examined the interactions between carrageenans and the gut microbiome in the pathogenesis of gastrointestinal disorders. This review argues for personalized guidance on carrageenan intake based on individuals' health status. Future research efforts that aim to close the knowledge gap on the effect of low-dose and chronic carrageenan intake as well

as interactions among food additives should be conducive to the improved safety profile of carrageenans in processed food products." As taken from Liu F et al. 2021. Rev. Food Sci. Food Saf. 20(4), 3918-3936. PubMed, 2021 available at https://pubmed.ncbi.nlm.nih.gov/34146449/

6. Functional effects on

6.1. Broncho/pulmonary system

"A transbronchial injection of 0.75% carrageenan in physiologic saline induced pneumonia followed by emphysema in the insulted lobe. In the stages of pneumonia, scattered infiltration of polymorphonuclear leukocytes was seen throughout the affected lobe within a few days of treatment; later this was replaced by the accumulation of carrageenan-laden macrophages, which lasted for one to two months. Enlargement of alveoli and alveolar ducts appeared 2 weeks to 2 months after the treatment, and pulmonary emphysema was observed at 4 months. The lobes that were not treated with carrageenan were normal in appearance during both the pneumonia and the emphysema. Morphometric analysis of the lung at 4 months showed decrease of the alveoli and/or alveolar ducts and enlargement of their luminal spaces, also suggesting the development of emphysema. In contrast to various kinds of elastases that are known to produce emphysematous changes in animals, the elastolytic activity of carrageenan solution did not show any such effects, although in the homogenate of the lobes given carrageenan, a moderate but significant increase in the proteinase activities of alveolar macrophages are said to occur (Bowers et al. 1985). It was suggested that carrageenan-induced emphysema is a chronic disorder associated with both carrageenan toxicity and accumulated carrageenan-laden macrophages in the insulted lobes." As taken from Mitsuhashi T et al. 1989. Tohoku J. Exp. Med. 157(2), 163-76. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/2711383

6.2. Cardiovascular system

"Bhattacharyya et al. (2012) investigated a possible relationship with markers related to the development of diabetes following observations on the interaction of carrageenan with TLR4, as systemic inflammation in Type 2 diabetes might be mediated by the TLR4 pathway. Groups of six male mice were administered carrageenan (commercial κ-λ-type, no indication on weight-average molecular weight was given.) in drinking- water at 0 or 10 µg/mL (equal to 1.7 mg/kg bw per day). A glucose tolerance test (GTT) and an insulin resistance test (ITT) were performed in the same mice at 12 and 14 weeks of age, respectively. No treatment-related effects on body weight or water consumption were observed. In the GTT, blood glucose was statistically significantly increased by carrageenan at all time points. In the ITT, blood glucose levels were statistically significant decreased by more than 80% in controls, but by only 43% in the carrageenan-treated mice, at all time points.

The Panel agreed with JECFA who noted a number of limitations in this study: 'the animals underwent an 18-h overnight fast, which is a considerable length of time for mice that are nocturnal feeders and is known to enhance reactions to insulin in mice (Ayala et al., 2010); measurements of blood glucose extended to only 90 min after dextrose injection rather than 120 min, which is recommended as one of the critical time points for comparisons between treated and control groups '(Ayala et al., 2010 as cited in JECFA, 2015).

According to the results of a 1-year study in mice (Bhattacharyya et al., 2015), exposure to carrageenan may lead to fasting hyperglycaemia and in combination with high fat diet exacerbation of glucose intolerance and hyperlipidemia without effect on weight. No indication about the weight-average molecular weight was given."

As taken from EFSA, 2018.

6.3. Nervous system

“INTRODUCTION: To better understand the pathophysiology of chronic muscle pain, there are multiple animal models that mimic different acute/chronic pain conditions, such as carrageenan injection. Our previous studies demonstrated differences between muscles of different innervation in acute pain. In this study we characterized the effect of carrageenan in 2 muscles: masseter (trigeminal innervation) and gastrocnemius (spinal innervation). METHODS: Carrageenan (3%, 6%, and 9%) was injected into the masseter and gastrocnemius of rats. Mechanical, heat, and chemical nociceptive thresholds were measured for 14 days. RESULTS: Carrageenan did not induce mechanical allodynia or thermal hypersensitivity in either muscle. Instead, it induced a short-lasting mechanical hyperalgesia, greater in the masseter than in the gastrocnemius. CONCLUSION: Carrageenan injected into the masseter and gastrocnemius induces a short-lasting hyperalgesia. These results could indicate a higher susceptibility of orofacial muscles to this type of insult and, consequently, a difference between trigeminal and spinal innervation.” As taken from Bagüés A et al. 2017. Muscle Nerve 56(4), 804-813. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/28026014

6.4. Other organ systems, dependent on the properties of the substance

“Three groups of three males and five females infant baboons (Papio cynocephalus) received infant formula containing nominal concentrations of 0, 300 and 1,500 mg carrageenan/l from birth to 112 days of age (McGill et al., 1977), being the estimated intake about 86 and 432 mg/kg bw per day. The test material was described as native carrageenan (no indication about weight-average molecular weight), it was heated in the formula and bound to protein to stabilise the suspension of fat, protein and other nutrients. Baboons were fed formula five times per day for the first 14 days, four times per day for the next 14 days, three times per day for the next 56 days and twice per day for 28 days until 112 days of age. No other food or fluid were given to the animals. The baboons were observed daily and the body weight was measured daily. Stools were checked weekly for occult blood. Blood samples were taken monthly, and organ weights were measured at necropsy. On the night before necropsy, each baboon was given water only ad libitum. At necropsy under anaesthesia, the GI tract was removed and fixed in 10% buffered formalin. The gut was observed minute-by-minute as fresh and after fixation. Samples were taken for both histological section and for microscopic analysis (from all major viscera, muscle, lymph nodes and the central nervous system). Microscopic examination of the colon showed reddening areas beneath the epithelium and to a lesser degree deeper in the lamina propia on which in few instances, deposits of granular brown pigment representing hemosiderin were reported. Occult blood in the faeces was also reported. Although the authors could not explain the cause or origin of the occult blood in faeces they concluded that it was not associated with carrageenan content of the formula. There were no effects on: organ or body weights, urine and faeces, haematological or clinical chemical variables. Hyperplasia of lymphatic tissue and the inflammatory lesions of the colon were present in all animals, regardless of the presence or concentration of carrageenan. The authors attributed these findings to possible antigenic stimulation by exogenous milk proteins.”

“A randomised, double-blind, placebo-controlled, multicenter, clinical trial was conducted to assess if patients with ulcerative colitis in remission would have a longer interval to relapse if they followed a diet without carrageenan (Bhattacharyya et al., 2017). No indication about the weight-average molecular weight of the tested material was given. It is reported that most of the food-grade carrageenan is comprised of κ- and λ-carrageenans of molecular weight 200–800 kDa. Twelve patients completed the study questionnaires. There was relapse in three patients who received carrageenan-containing capsules. None of the patients who received placebo-containing capsules relapsed ($p = 0.046$, log-rank test). Laboratory tests showed increases in faecal calprotectin ($p = 0.06$; paired t-test, two-tailed) and interleukin-6 ($p = 0.02$, paired t-test, two-tailed) between the beginning and the end of study participation in the carrageenan-exposed group, but not in the placebo group. The authors concluded that carrageenan intake contributed to an earlier relapse in patients with ulcerative colitis in remission. The Panel noted that the study is of limited relevance due to the low number of patients involved.”

As taken from EFSA, 2018.

“The ability of edible gums to depress total liver lipids and activities of two hepatic enzymes (glucose-6-phosphate dehydrogenase and NADP-linked malic enzyme) was examined during the refeeding of 2 d starved rats. Gums were fed as 4% of dry ingredients, occasionally with added water, in otherwise identical high glucose, nutritionally adequate diets. Feeding of xantham gum for 1 or 2 d decreased the rise in two enzyme activities and in total liver lipids, but after 4 or 7 d only total liver lipids were affected. Agar, which is insoluble at room temperature, was effective only when incorporated in the diet as a stiff gel. Guar, carrageenan, karaya and pectin reduced the change in at least one hepatic parameter, but acacia and gum ghatti, which impart little viscosity to water, were without effect. The ability of seven gums to form semisolid gels at low concentrations was measured and appeared to be associated with the variable effectiveness of those gums in influencing lipid contents and enzyme activities of the liver.” As taken from Osilesi O et al. 1988. J. Nutr. 118(4),462-468. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/2833589

“BACKGROUND: Orthodontic tooth movement (OTM) can be accelerated by increasing bone turnover. Carrageenan is a common food additive, which can induce inflammation. Hence, it might accelerate OTM. However, it has not been investigated to date. METHODS: This 2-phase preliminary animal experimental study was conducted on 28 Wistar rats. A pilot study on 5 mice was done to estimate the experimental dose of carrageenan. The first phase evaluated the effect of a 40μL carrageenan 1% injection on inflammation status in 7 rats versus 7 control rats, 6hours after the injection. The second phase examined the effect of the same dose of carrageenan on OTM speed during 21 days of incisor retraction in rat, in two groups: control (normal saline) and carrageenan (n=7×2). This sample size was pre-determined based on a priori power calculations. In phase II, osteoclastic activity was also evaluated, 21 days after the injection. The groups were compared statistically (α=0.05). RESULTS: Six hours after carrageenan injection, 1, 3, and 3 rats had inflammation scores of 3 to 1, respectively. Six hours after saline injection, 1 rat had a score of 1 and the remainder had no inflammation (P=0.0023, Mann-Whitney). Twenty-one days after saline and carrageenan injection, OTMs were 0.7±0.3 and 1.1±0.4mm, respectively, (P=0.053, Mann-Whitney). Twenty-one days after saline and carrageenan injection, mean osteoclast counts were, respectively, 4.87±1.849 and 7.143±1.727 per field (P=0.025, Mann-Whitney). CONCLUSION: Local injection of carrageenan can induce inflammation after 6hours. It can increase approximately 1.6-fold the speed of OTM, and increase the osteoclast count 1.5-fold after 21 days of space closure.” As taken from Kavoli S et al. 2017. Int Orthod. 15(4), 588-599. PubMed, 2018 available at: https://www.ncbi.nlm.nih.gov/pubmed/29128199

“Local muscle inflammation was induced by injecting carrageenan (10 mg/kg) into the right tibialis anterior muscle in 22 healthy ARC mice (6 weeks old). The contralateral muscle was injected with sterile isotonic saline, and the muscles were removed after 24 h for measurement of contractile function and cytokine concentration. Carrageenan significantly reduced maximum specific force, decreased the maximum rate of force development, altered the force-frequency relationship, and increased intramuscular levels of pro-inflammatory cytokines and chemokines. These results indicate that injected carrageenan directly affects contractile function and causes skeletal muscle weakness.”

As taken from CIR, 2015.

7. Addiction

JTI is not aware of any information that demonstrates that this ingredient has any addictive effect” if no information available.

8. Burnt ingredient toxicity

Endpoint	Tested level (ppm)	Reference
Smoke chemistry	20.000	Coggins CR et al. 2011b
In vitro genotoxicity	20.000	Coggins CR et al. 2011b
In vitro cytotoxicity	20.000	Coggins CR et al. 2011b
Inhalation study	20.000	Coggins CR et al. 2011b

9. Heated/vapor emissions toxicity

No data available to us at this time.

10. Ecotoxicity

10.1. Environmental fate

No data provided for this section.

10.2. Aquatic toxicity

No data provided for this section.

10.3. Sediment toxicity

No data provided for this section.

10.4. Terrestrial toxicity

No data provided for this section.

10.5. All other relevant types of ecotoxicity

No data provided for this section.

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12. Other information

No data available to us at this time.

13. Last audited

September 2022

Safety Assessment of Microbial Polysaccharide Gums as Used in Cosmetics

Status:	Final Report for public distribution
Release Date:	October 5, 2012
Panel Meeting Date:	September 10-11, 2012

The 2012 Cosmetic Ingredient Review Expert Panel members are: Chairman, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; Ronald A. Hill, Ph.D.; Curtis D. Klaassen, Ph.D.; Daniel C. Liebler, Ph.D.; James G. Marks, Jr., M.D., Ronald C. Shank, Ph.D.; Thomas J. Slaga, Ph.D.; and Paul W. Snyder, D.V.M., Ph.D. The CIR Director is F. Alan Andersen, Ph.D. This report was prepared by Monice M. Fiume, Senior Scientific Analyst/Writer, and Bart A. Heldreth, Ph.D., Chemist, CIR.

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ABSTRACT

The CIR Expert Panel assessed the safety of 34 microbial polysaccharide gums for use in cosmetics, finding that these ingredients are safe in cosmetic formulations in the present practices of use and concentration. The microbial polysaccharide gums named in this report have a variety of reported functions in cosmetics, including emulsion stabilizer, film former, binder, viscosity increasing agent, and skin conditioning agent. The Panel reviewed available animal and clinical data in making its determination of safety.

INTRODUCTION

This assessment is a review of information relevant to the safety of 34 microbial polysaccharide gums for use in cosmetic formulations. Reported functions for these ingredients include emulsion stabilizer, film former, binder, viscosity increasing agent, and skin conditioning agent.

The Cosmetic Ingredient Review (CIR) Expert Panel has reviewed other non-microbial gums and polysaccharides. In 2012, CIR concluded the Galactomannans, a group of 16 legume polysaccharides, are safe as used in cosmetics.[1] In 2009, the CIR Expert Panel reviewed the safety of Hyaluronic Acid, an amine-derived exopolysaccharide, finding it safe as used.[2] In 1987, the Expert Panel reviewed the safety of Tragacanth Gum (now named Astragalus Gummifer Gum), and concluded that Tragacanth Gum was safe as used; the Panel reaffirmed that conclusion in 2006.[3] Other plant-derived gums that the Panel has reviewed include Acacia Catechu Gum, Acacia Farnesiana Gum, Acacia Senegal Gum; in 2005, the Panel concluded that Acacia Senegal Gum is safe as used, but that the data are insufficient to support the safety of Acacia Catechu Gum and Acacia Farnesiana Gum as used in cosmetics.[4]

Three ingredients included in this safety assessment are hydroxypropyltrimonium chloride compounds. Although hydroxypropyltrimonium chloride itself is not a cosmetic ingredient, it is structurally analogous to some of the compounds included in the CIR safety assessment on trimoniums, in which the CIR concluded that the trimoniums are safe as used in cosmetics when formulated to be non-irritating.[5]

The 34 microbially-produced polysaccharide gums included in this review are:

- Xanthan Gum
- Hydroxypropyl Xanthan Gum
- Undecylenoyl Xanthan Gum
- Dehydroxanthan Gum
- Xanthan Gum Crosspolymer
- Xanthan Hydroxypropyltrimonium Chloride
- Gellan Gum
- Welan Gum
- Biosaccharide Gum-1
- Biosaccharide Gum-2
- Biosaccharide Gum-3
- Biosaccharide Gum-4
- Biosaccharide Gum-5
- Pseudoalteromonas Exopolysaccharides
- Dextran
- Carboxymethyl Dextran
- Dextran Hydroxypropyltrimonium Chloride
- Sodium Carboxymethyl Dextran
- Dextran Sulfate
- Sodium Dextran Sulfate
- Sclerotium Gum
- Hydrolyzed Sclerotium Gum
- Beta-Glucan
- Beta-Glucan Hydroxypropyltrimonium Chloride
- Beta-Glucan Palmitate
- Hydrolyzed Beta-Glucan
- Oxidized Beta-Glucan
- Sodium Carboxymethyl Beta-Glucan
- Pullulan
- Myristoyl Pullulan
- Levan
- Rhizobian Gum
- Hydrolyzed Rhizobian Gum
- Alcaligenes Polysaccharides

Some of the polysaccharide gums discussed in this safety assessment can be produced by more than one organism, and sometimes by plants. For example, beta-glucans are produced by fungi, yeasts, and grains[6] and levan can be produced by bacteria, yeasts, or fungi.[7]

Many studies have been conducted with some of the microbial polysaccharide gums in regard to health claims, immunomodulatory activity, anti-oxidant activity, etc. This safety assessment includes only studies and study-types that relate directly to the safety of the cosmetic use of these ingredients.

CHEMISTRY

Definition and Structure

Microbial polysaccharide gums are high molecular weight (MW) carbohydrate polymers that make up a substantial component of the cellular polymers found in and surrounding most microbial cells.[8] These polysaccharide gums are produced by a wide variety of microorganisms and are water soluble gums that have novel and unique physical properties. Microbial polysaccharide gums are generally divided into three groups: exocellular, cell wall, and intercellular.[9] The exocellular polysaccharide gums are those that

constantly diffuse into the cell culture medium and are easily isolated. The cell wall (i.e., structural) and intercellular polysaccharide gums are integral parts of the cell wall or capsular products, and are more difficult to separate from cell biomass.

Microbial polysaccharide gums may be ionic or non-ionic and are primarily linear polysaccharides to which side-chains of varying length and complexity are attached at regular intervals.[8] Most microbial polysaccharide gums are linear hetero-polysaccharides consisting of three to seven different monosaccharides arranged in groups of 10 or less to form repeating units. The monosaccharides may be pentoses, hexoses, amino sugars, or uronic acids. For example, xanthan gum is a polysaccharide produced by a pure-culture fermentation of a carbohydrate with *Xanthomonas campestris*, and is composed of glucose, glucuronic acid, 6-acetylmannose, and 4,6-pyruvylated mannose residues, as seen in Figures 1 and 2.

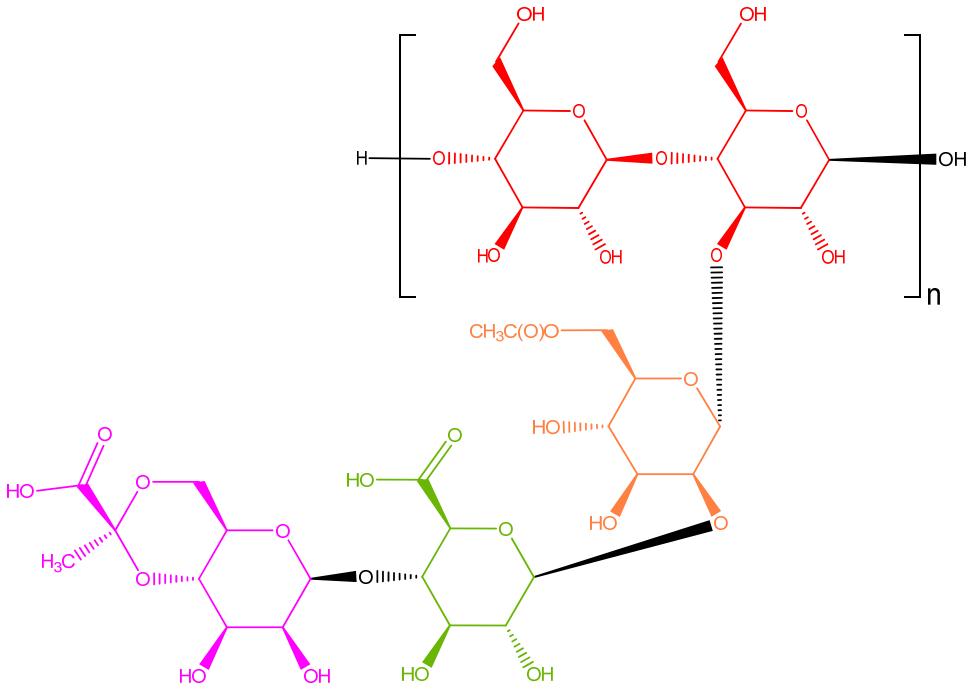


Figure 1. Xanthan Gum – a polysaccharide composed of glucose, glucuronic acid, 6-acetylmannose, and 4,6-pyruvylated mannose

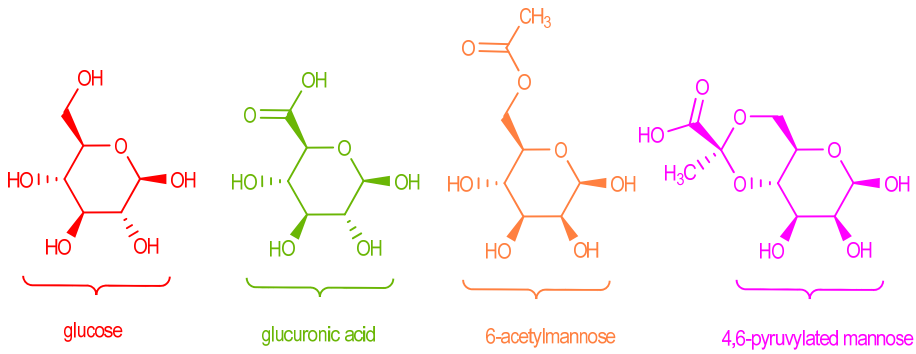


Figure 2. Glucose, glucuronic acid, 6-acetylmannose, and 4,6-pyruvylated mannose, the monosaccharide components of Xanthan Gum.

The other ingredients in this report are related by having similar polymeric repeat units. The definitions and polymeric repeat units of the ingredients included in this review are provided in Table 1.

Physical and Chemical Properties

Available physical and chemical properties are provided in Table 2. The properties of the microbial polysaccharide gums can vary widely based on, among other parameters, the side groups, the ester substituents, or the bacterial strains, but generally speaking, these are very large MW polymers.[10-13]

Constituents/Impurities

The available constituent and impurity data are provided in Table 3.

Method of Manufacture

Methods of manufacture for many of the microbial polysaccharide gums are provided in Table 4. Some of the polysaccharide gums discussed in this safety assessment can be produced by more than one organism, and in some cases, by plants. For example, beta-glucans are produced by fungi, yeasts, and grains[6] and levan can be produced by bacteria, yeasts, or fungi.[7]

USE

Cosmetic

The microbial polysaccharide gums named in this report have a variety of reported functions in cosmetics that include emulsion stabilizer, film former, binder, viscosity increasing agent, and skin conditioning agent.[14] The Food and Drug Administration (FDA) collects information from manufacturers on the use of individual ingredients in cosmetics as a function of cosmetic product category in its Voluntary Cosmetic Registration Program (VCRP). VCRP data obtained from the FDA in 2012,[15] and data received in response to a survey of the maximum reported use concentration by category conducted by the Personal Care Products Council (Council),[16,17] indicate that 19 of the 34 microbial polysaccharide gums named in this safety assessment are currently used in cosmetic formulations. Xanthan gum is used in almost every category of cosmetic product, with 3470 reported uses. Biosaccharide gum-1, sclerotium gum, and beta-glucan are reported to be used in 346, 193, and 137 cosmetic formulations, respectively. All other in-use ingredients have less than 70 uses. The ingredient with the highest concentration of use is pullulan; it is used at up to 12% in leave-on formulations (i.e. tonics, dressings, and other hair grooming aids) and 17% in "other" oral hygiene products (a breath freshener that dissolved in the mouth[18]). Both xanthan gum and biosaccharide gum-1 are used at up to 6% in leave-on formulations and xanthan gum crosspolymer and biosaccharide gum-4 are used at 5% in leave-on formulations. All other in-use ingredients are used at concentrations of ≤3%.

In some cases, reports of uses were received in the VCRP, but no concentration of use is available. For example, sodium carboxymethyl dextran is reported to be used in 10 formulations, but no use concentration data were available. In other cases, no reported uses were received in the VCRP, but a use concentration was provided in the industry survey. For example, hydrolyzed sclerotium gum was not reported in the VCRP to be in use, but the industry survey indicated that it is used in leave-on formulations at up to 1%. It should be presumed that hydrolyzed sclerotium gum is used in at least one cosmetic formulation.

Frequency and concentration of use data are provided in Table 5. The ingredients not listed in the VCRP or by the Council as being used are listed in Table 6.

Products containing some of the microbial polysaccharide gums are reported to be used on baby skin, to be applied to the eye area or mucous membranes, or could possibly be ingested. Some of these ingredients are reported to be used in product types that may be inhaled; for example, dehydroxanthan gum is used in a face and neck spray at 0.2%. In practice, 95% to 99% of the particles released from cosmetic sprays have aerodynamic equivalent diameters in the 10 to 110 μm range.[19,20] Therefore, most particles incidentally inhaled from these sprays are deposited in the nasopharyngeal region and are not respirable.[21,22] Xanthan gum is reported to be used in deodorants at up to 0.6%, and it is not known whether or not these products are sprayed. There is some evidence indicating that deodorant spray products can release substantially larger fractions of particulates having aerodynamic diameters in the range considered to be respirable.[22] However, the information is not sufficient to determine whether significantly greater lung exposures result from the use of deodorant sprays, compared to other cosmetic sprays.

All of the microbial polysaccharide gums named in the report are listed in the European Union inventory of cosmetic ingredients.[23]

Non-Cosmetic

Non-cosmetic uses are of microbial polysaccharide gums are summarized in Table 7. Some of the food and medical use information are described in the following paragraphs.

Xanthan gum[24] and gellan gum[25] are approved as direct food additives in gums, chewing gum bases, and related substances. Xanthan gum is also approved as an indirect food additive.[26] Beta-glucan (as curdlan, a specific beta-glucan that is a linear polymer consisting of β-(1→3)-linked glucoside residues) is approved as a direct food additive for multipurpose addition.[27] Dextran is an indirect food additive that is generally recognized as safe (GRAS).[28] The World Health Organization (WHO) concluded that studies on the safety of xanthan gum,[29] gellan gum,[30] and pullulan[31] provided sufficient information to be allocated an acceptable daily intake (ADI) of "not specified". Pullulan appears in the Japanese List of Existing Food Additives.[32]

Xanthan gum is used as a stabilizer, thickener, and emulsifying agent in water-based pharmaceutical preparations.[33] Dextran, as dextran 70, is an approved active ingredient for over-the-counter (OTC) use as an ophthalmic demulcent at 0.1% when used with another approved polymeric demulcent.[34] Dextran is used as a plasma volume expander (as dextran 70) and as a blood flow adjuvant (as dextran 40).[35] Sodium dextran sulfate is used as a clinical reagent.

TOXICOKINETICS

Absorption, Distribution, Metabolism, and Excretion

Dermal

In Vitro

Beta-Glucan

A single application of 5 mg/cm^2 of a 0.5% (oat) beta-glucan solution was applied to human abdominal skin.[36] Beta-glucan penetrated the skin into the epidermis and dermis. (No details were provided.)

Human

Dextran

Fluorescent dextrans (FDs) in aqueous (aq.) solution were used to determine the dermal absorption of different MW dextrans (MW 3000 - 70,000, identified as FD-3 and FD-70, respectively) through skin that has been subjected to mini-erosion.[37] Prior to testing, absorption of FD-20 from a test "Cellpatch" (cell) into systemic circulation via a mini-erosion site was verified in two male subjects and one female subject. The largest of the test molecules filtered from the blood into the urine was 20,000 MW, and with increasing erosion diameter, absorption increasingly occurs via the lymphatic system. Each subject received a single cell containing 0.5 ml solution (5 mmol). After 24 h, a mean of 54.3% of the total FD-20 dose had been absorbed, and 12.6% of the absorbed dose was recovered in the urine.

The effect of molecular size on absorption of FDs was then determined. Four cells with 100 µl of FD-3, FD-10, FD-20, or FD-70 in 0.5 ml isotonic saline were applied for 24 h to 6 mm mini-eroded sites on 4 male and 3 female subjects. A fifth cell, without FD, was applied as a negative control. The FD concentration in each cell was measured using spectrofluorometry at various times. The dextrans were readily absorbed, but absorption decreased with increasing molecular size. The absorption of FD-3 was 37.9%, but for FD-70, it was 20.1%. Further testing using 3 male and 3 female subjects determined that the degree of transdermal absorption was directly related to the area of erosion; 20.5% of FD-3 absorbed through a 3 mm erosion area, while 60.7% of the same molecular size FD absorbed through a 10 mm erosion.

Oral

Non-Human

Xanthan Gum

Rats were fed a diet containing 2% [^{14}C]xanthan gum that was produced by fermentation of uniformly-labeled glucose with *Xanthomonas campestris*.[38] No accumulation was found in the tissues. A maximum of 15% of the radioactivity was metabolized to carbon dioxide within 100 h. Fecal analysis indicated that there was no accumulation of the polysaccharide material, except acetate. (Acetate and pyruvate accounted for only 9.8% of the label in the gum used). In the feces, 98% of the radioactivity was attributed to unchanged or slightly modified polysaccharide. In vitro testing indicated that non-enzymatic hydrolysis and fecal microorganisms were responsible for the in vivo breakdown of xanthan gum. (No additional details were provided.)

Gellan Gum

In animal feeding studies using radiolabeled gellan gum, the majority of the gellan gum that was administered was recovered in fecal matter.[13] This appears to indicate that no endogenous enzymes that are able to break down gellan gum are present in the small intestine. (No additional details were provided.)

The absorption, distribution, and excretion of gellan gum was determined in studies using a dually-radiolabeled gum that was prepared in separate fermentations using [^{3}H]glucose and [^{14}C]glucose as carbon sources.[39] (The ^{3}H product was subjected to multistage purifications for a relatively pure [^{3}H]polysaccharide, which was then added to the ^{14}C fermentation, giving a polysaccharide fraction that was dual-labeled and a non-polysaccharide fraction labeled only with $^{14}CO_2$.) In the first study, one male and one female Sprague-Dawley rat were dosed by gavage with a single dose of 960 mg/kg [^{3}H/^{14}C]gellan gum (4 µCi). Expired air was collected for 24 h after dosing, and <0.55% of the dosed radioactivity was detected as ^{14}C.

Four male and three female Sprague-Dawley rats were then given a single dose by gavage of 870 mg/kg [^{3}H/^{14}C]gellan gum (2.9-4.1 µCi ^{14}C; 0.7-0.9 µCi ^{3}H). Urine and feces were collected for 7 days. Approximately 86% of the dosed ^{14}C was excreted in the feces and 2-3% in the urine, and approximately 100% of the dosed ^{3}H was excreted in the feces and 4% was in the urine. Tissue and carcass ^{14}C radioactivity was approximately 3-4% of the dose. The ^{3}H activities in the tissues were below the limits of accurate quantification.

In the last study, one male and four female Sprague-Dawley rats were dosed with 1 g/kg [^{3}H/^{14}C]gellan gum by gavage, and blood samples were taken at various intervals over a 7-day period. (It is not stated, but it is appears that one dose was administered). The peak level of radioactivity in blood, equivalent to 0.4% of the dose, occurred about 5 h after dosing.

Dextran

Groups of 5 fasted male Sprague-Dawley rats were given a single dose by gavage of 50 mg/kg fluorescein-labeled dextrans (FD-4, 4400 avg. MW; FD-20, 19,000 avg. MW; or FD-40, 40,500 avg. MW).[40] The dextran solution was prepared as 25 mg/ml in isotonic phosphate buffer. Blood samples were taken at various intervals for up to 4 h after dosing with FD-4, up to 8 h after dosing with FD-20, and for up to 24 h with FD-40. Urine samples were taken at intervals for up to 8 h after dosing with FD-4 and FD-20 and for up to 24 h after dosing with FD-40. None of the dextrans were detected in the serum after oral administration. Small amounts of the dose, ranging from 0.308% with FD-4 to 0.0138% FD-40, were detected in the urine. The oral bioavailability was 0.398, 0.0728, and 0.0431% for FD-4, FD-20, and FD-40, respectively.

Dextran Sulfate

Two groups of 5 male Wistar rats were dosed by gavage with 5 mg/ml of 20 mg/kg dextran sulfate containing 12 µCi ^{3}H-dextran sulfate/kg (8000 avg. MW), and each group was killed 3 or 24 h after dosing.[41] Most of the radioactivity was detected in the feces; 22.5% of the dose was recovered after 24 h. Metabolites, breakdown products, and $^{3}H_2O$ were not found in the feces, and the researchers stated that it was most likely that the dose recovered in the feces was unabsorbed dextran sulfate. Approximately 10% of the dose was recovered in the urine after 24 h, with 6% recovered after 3 h. Urinary ^{3}H elution profiles indicated that intermediate MW metabolites or breakdown products were formed and that either intact or partially intact dextran sulfate was absorbed through the epithelium of the gastrointestinal (GI) tract. The 6-24 h samples indicated a marked shift towards smaller MW products.

Beta-Glucan

Two male Sprague-Dawley rats were dosed orally with 20 mg/kg bw [U-^{14}C]beta-glucan (as curdlan) in water prepared from [U-^{14}C]glucose.[42] Most of the radioactivity was recovered in expired CO_2; 77% of the dose was recovered in 24 h and 89% in 72 h. A total of 7.7 and 12% of the radioactivity as administered dose was recovered in the feces after 24 and 72 h, respectively, and 2.6 and 3.3% was recovered in the urine after 24 and 72 h, respectively.

In another study, three male Wistar rats were dosed orally with 20 mg/kg bw [^{14}C] beta-glucan (as curdlan) in water.[42] Initially (i.e. the first 3 h), the excretion of $^{14}CO_2$ was low, but then increased linearly up to 12 h, plateauing at 39% of the administered radiolabel. A total of 3.4 and 3.8% of the radioactivity as administered dose was recovered in the feces after 24 and 48 h, respectively, and 1.3 and 1.4% was recovered in the urine after 24 and 48 h, respectively. In a group of three male Wistar rats, administered 5 mg/ml tetracycline for 5 days prior to and 2 days following beta-glucan, it was demonstrated that the intestinal microflora are partly responsible for the metabolism of beta-glucan to carbon dioxide.

The effect of dose on metabolism was also examined.[42] Three male Wistar rats were given an oral dose of 2.3, 23, or 230 mg/kg bw [^{14}C]beta-glucan (as curdlan) in water. At the two higher doses, excretion of radioactivity as carbon dioxide decreased with increasing dose, while fecal excretion of the radiolabel increased. The researchers stated that this was an indication of limited metabolism at higher doses.

Pullulan

Five fasted male Wistar rats were dosed by gavage with a 2 ml of a 10% solution of pullulan (49,000 MW) in 0.9% saline.[43,44] The animals were killed 1 h after dosing and the contents of their stomach and small intestines were collected. Approximately 3% of the pullulan had been hydrolyzed; it was not known whether the hydrolysis products were absorbed by the small intestine.

Human

Dextran

Dextran can be depolymerized by α-1-glycosidases (dextranases) that occur in the liver, spleen, kidney, and lower part of the GI tract.[10]

Dextran Sulfate

Six fasted male subjects were given a single oral dose of 1800 mg dextran sulfate (7000-8000 MW; 17-20% sulfur) and, after 48 h, a single intravenous (i.v.) dose of 225 mg dextran sulfate in saline infused over 60 min.[45] After oral dosing, no measurable dextran sulfate was found in the plasma using the competitive binding assay, and there was no increase in activated partial thromboplastin time (APTT). Plasma lipolytic activity did not increase the first 3 h after oral dosing; at 3-4 h after oral dosing, it increased by two times the baseline average. Very little dextran sulfate was recovered in the urine after oral dosing. After i.v. dosing, peak plasma concentrations were 26-35 µg/ml, and the APTT was increased by an average of 6.9 times over the baseline values. The plasma lipolytic activity increased by an avg. of 438 times the baseline value. Dextran sulfate was recovered in the urine after i.v. dosing.

Pullulan

Pullulan is only partially hydrolyzed by salivary and pancreatic amylases of the upper GI tract; essentially no monomeric glucose is released during hydrolysis.[44] Pullulan is largely resistant to digestion in the GI tract because of the occasional presence of 1→3-glycosidic linkages and the high percentage of α-1→6-glycosidic linkages.[31] The degree of digestion appears to be dependent on

molecular mass. Pullulan is fermented in the colon by intestinal microflora to produce short-chain fatty acids; the degree of fermentation is dependent on the degree of polymerization of pullulan.

Six subjects ingested 10 g pullulan (50,000 MW) for 14 days.[44,46] Administered pullulan was fully digested in the intestinal tract and was not detected in the feces. After 14 days, the fecal short chain fatty acid concentration increased from 6 mg/g to 8.8 mg/g. The researchers concluded that pullulan was completely fermented to short-chain fatty acids by intestinal bacteria.

Parenteral

Non-Human

Dextran

Rabbits were given a daily i.v. 30 ml dose of a 6% solution of partly hydrolysed bacterial dextran (75,000 avg MW) 6 days/wk for 103-113 wks.[47] Eleven animals were evaluated. Approximately 25% of the dose was excreted in the urine. The plasma concentration of dextran at study termination (0.50 g/100 ml) did not differ from the value at 2 mos (0.44 g/100 ml), but it was generally greater than the value reported at 24 h after a single 30 ml dose (not given). Moderate dextran storage was observed in the spleen without an increase in the total carbohydrates. However, considerable storage was observed in the liver with a marked increase in carbohydrates. (Additional details and results are provided in the section on 'Repeated Dose Toxicity').

Groups of 5 male Sprague-Dawley rats were given a single i.v. dose of 5 mg/kg FD-4 (4400 avg. MW), FD-20 (19,000 avg. MW), FD-40 (40,500 avg. MW), FD-70 (71,000 avg. MW), or FD-150 (147,800 avg. MW).[40] The dextran solution was prepared as 5 mg/ml in isotonic phosphate buffer. Blood samples were taken at various intervals for up to 4 h after dosing with FD-4, up to 8 h after dosing with FD-20, and for up to 24 h with FD-40, FD-70, and FD-150. Urine samples were taken at intervals for up to 8 h after dosing with FD-4 and FD-20 and for up to 24 h after dosing with FD-40, FD-70, and FD-150. Pharmacokinetic parameters were MW-dependent. Concentrations of the three highest MW dextrans could be detected in serum for up to 12 h after dosing, while FD-20 and FD-4 were not found in the serum after 3 and 1.5 h, respectively. The distribution half-life ($t_{1/2}\alpha$) ranged from 0.0517 to 0.895 h for FD-4 and FD-150, respectively, and the elimination half-life ($t_{1/2}\beta$) ranged from 0.282-3.03 h for FD-4 and FD-150, respectively.

Male Sprague-Dawley rats were also given a single i.v. dose of 1, 25, or 100 mg/kg FD-4 (avg. MW 4300) and FD-150 (avg. MW 145,000).[48] The dextran solution was prepared as isotonic phosphate buffer at a concentration that would result in a test volume of 2 ml/kg. Groups of 4 rats each were used for blood, urine, and tissue samples at various intervals for up to 6 h in rats dosed with FD-4 and for up to 96 h in rats dosed with FD-150. Renal excretion was a major excretion pathway for FD-4 but not FD-150. Urinary recovery ranged from 79-82% of the dose with FD-4 and only from 1.1-2.1% of the dose with FD-150; at each MW, the dose administered did not have a statistically significant effect on the amount excreted. Renal clearance ranged from 344-360 ml/h/kg with FD-4 and from 0.131-0.245 ml/h/kg for FD-150, and systemic clearance ranged from 420-457 ml/h/kg for FD-4 and from 8-20 ml/k/kg for FD-150. The highest concentrations of FD-4 were found in the kidneys at 1 min after dosing (9.31%, 10.0%, and 10.4% of the dose with 1, 25, and 100 mg/kg, respectively) was linear with dose. The highest concentrations of FD-150 were found in the liver (68.5% at 5 h, 51.6% at 24 h, and 41.5% of the dose at 24 h with 1, 25, and 100 mg/kg, respectively) and the spleen (11.5% at 12 h, 2.09% at 48 h, and 1.21% of the dose at 96 h with 1, 25, and 100 mg/kg, respectively); recovery of dextran in the liver and spleen was non-linear, with a greater difference seen in the spleen than in the liver. The researchers reported that the MW of recovered FD-4 remained relatively constant, but that of recovered FD-150 changed significantly. The MW of FD-150 recovered in the urine was <40,000, and, in the liver, the avg. MW at 96 h was ~70,000. The estimated MW of the recovered FD-150 in the liver appeared to be dose-dependent with higher doses having a higher avg. MW. The researchers concluded that excretion of lower MW dextrans was independent of dose, while excretion of higher MW dextrans was dose-dependent.

Another study also found that MW affected the distribution and excretion of dextran.[49] Female BALB/cCrSlc mice were dosed i.v. with 0.1 ml of 0.1 wt% ^{125}I-labeled dextran, MW ranging from 4980-220,000, in phosphate buffered saline (PBS); dextran was labeled through radioiodination of tyramine residues. Blood samples were taken at various intervals for some groups, and tissues were collected from others. (The number of animals/group was not specified.) High MW dextran remained in the blood longer than lower MW dextran. The $t_{1/2}\beta$ also increased with increasing MW, with a pronounced change occurring around 30,000 MW. After 3 h, 83% of the dose of the lowest MW dextran was excreted, while only 41% of the highest MW dextran was found in excrement. Most of the high MW dextran was found in the liver; after 3 h, 5.2% of the high MW dextran was found in the liver, while only 0.7% of the lowest MW was recovered in that tissue. At 3 h, 3.5 vs. 19% of the dose of the lowest and highest MW dextran, respectively, was recovered in the carcass. Two to 10% of the dose was recovered in the GI tract, but the amount recovered did not appear to be related to MW.

Dextran Sulfate

A preliminary study was performed in which 2 male Wistar rats were dosed by i.v. injection with 20 mg/kg dextran sulfate 8000 avg. MW) containing 10 μCi [^{3}H]dextran sulfate/kg via the penile vein (5 mg/100 μl).[41] The rats were killed 1 or 3 h after dosing. The total ^{3}H excreted in the urine accounted for approximately 50% of the administered dose. Within 1 h after i.v. administration, rapid excretion of intact dextran sulfate occurred.

In the main study, groups of 5 male Wistar rats were dosed by i.v. injection via the penile vein with 5 mg/100 µl of 20 mg/kg dextran sulfate containing 12 µCi [^{3}H]dextran sulfate/kg, and each group was killed 3 or 24 h after dosing. Urine was the major route of excretion following i.v. dosing, with approximately 46% of the dose excreted within 3 h and 51% within 24 h. Approximately 2% of the dose was recovered in the feces after 24 h. Based on the ^{3}H elution profiles, it was hypothesized that dextran sulfate or its metabolites were incorporated into higher MW compounds, such as glycogen, at 3-6 h and smaller MW at 6-24 h. In the plasma, the highest concentration of dextran sulfate was found at 3 h; the amount decreased with time.

In the tissues, the highest amounts of radioactivity were distributed in the liver, kidney, and spleen. The amount of radioactivity recovered in the tissues following i.v. administration was compared to that found following oral administration. (The oral study was described previously in this safety assessment.) Concentrations of ^{3}H in all tissues and fluids were statistically significantly higher in the animals dosed by i.v. injection compared to those in animals dosed orally.

Beta-Glucan

Groups of two male Sprague-Dawley rats were dosed by intraperitoneal (i.p.) injections with 20 mg/kg bw [^{14}C]beta-glucan (as curdlan) in water, and the animals were killed 0.5, 3, 6, or 24 h after dosing.[42] After 24 and 48 h, only 1.8% and 4.1% of the radioactivity was recovered in CO_2, respectively, 0.05 and 0.12% was recovered in the feces, respectively, and 3.5 and 4.1% of the radioactivity as % of dose was recovered in the urine, respectively. Whole-body radioautography showed that the radiolabel was distributed in the intestinal fluids.

Pullulan

The effect of MW on the distribution and excretion of pullulan was examined.[49] Female BALB/cCrSlc mice were dosed i.v. with 0.1 ml of 0.1 wt% [^{125}I]pullulan, MW ranging from 5800-853,000, in PBS; pullulan was labeled through radioiodination of tyramine residues. Blood samples were taken at various intervals for some groups, and tissues were collected from others. (The number of animals/group was not specified.) High MW pullulan remained in the blood longer than lower MW pullulan. The $t_{1/2}\beta$ also increased with increasing MW. After 3 h, 96% of the dose of the lowest MW pullulan was excreted, while only 15% of the highest MW pullulan was found in excrement. At 3h, most of the high MW pullulan, 50% of the dose, was recovered in the liver; only 1% of the dose of the lowest MW pullulan was recovered in the liver after 3 h. The highest percentage of the dose recovered in the GI tract and the carcass was found with mid-level MW pullulan; 5.3% of the dose was recovered in the GI tract with 100,000 MW pullulan and 10.1% of the dose was recovered in the carcass with 48,000 MW pullulan.

Fasted male Wistar rats (number not specified) were injected with a single i.v. dose 2 ml of 0, 6, 12, 18, or 24 mg/kg fluorescein-labeled pullulan (MW 58,200) in saline in the jugular vein.[50] Pullulan was rapidly eliminated from the blood; however, elimination decreased as dose increased. Hepatic uptake was also dose-dependent; the hepatic uptake clearance of pullulan decreased with increased dose. In the liver, distribution of pullulan in the parenchymal cells was 2.5 times greater than in the nonparenchymal cells. The researchers did state that with a higher MW pullulan, avg. 70,000 MW, uptake was greater in the nonparenchymal cells.

Human

Dextran

Four male and three female subjects received an i.v. injection 100 ml a partially degraded dextran (40,000 avg. MW; 10% w/v in 0.9% saline).[51] Blood samples were taken at various intervals after dosing. There was an initial rapid decrease in the serum in the first hour after dosing. Different fractions of the dextran were eliminated from the plasma at different rates; higher MW fractions remained in the plasma longer.

TOXICOLOGICAL STUDIES

Single Dose (Acute) Toxicity

Acute toxicity studies are summarized in Table 8. The acute toxicity of xanthan gum, gellan gum, beta-glucan, sodium carboxymethyl beta-glucan, and pullulan was assessed orally in mice, rats, and/or dogs, and dextran sulfate and beta-glucan were tested by i.p. and i.v. dosing in mice and rats. There was no notable toxicity observed in these studies. In acute inhalation studies, the LC_{50} of xanthan gum was >21 mg/l in rabbits and of gellan gum was >5.06 mg/l in rats. A single 180-min exposure of humans office dust containing 10 mg curdlan/g dust resulted in decreased nasal volume, in swelling in the nasal turbinates, and an increase in nasal eosinophils when compared to "clean" dust.

Inflammatory Response

Beta-Glucan

The inflammatory response following a single exposure to beta-glucan (as curdlan) was evaluated in guinea pigs.[52-54] Mostly, no effect or a slight decrease in inflammatory cells in lung lavage was observed. A 4 h inhalation exposure to 1% beta-glucan in dust (mass mean aerodynamic diameter [MMAD] 5 µm) by guinea pigs produced a delayed subacute nasal congestion when compared to dust without beta-glucan (MMAD 6.5 µm) and resulted in decreased nasal volume; after 18 h, there was a significant decrease

in nasal volume.[52] In humans, inhalation exposure to beta-glucan in dust for four 3 h exposures resulted in increased nasal swelling and decreased nasal volume and an immediate increased in nasal eosinophil/ml count.[55]

Pullulan

The effect of pullulan on inflammation was investigated in ICR mice using the xylene-induced acute inflammatory mouse ear model.[56] Groups of nine mice were dosed orally with 0, 62.5, 125, or 250 mg/kg pullulan in distilled water 30 min prior to topical application of xylene to one ear. Two h after xylene application, all animals were killed. Compared to xylene-treated controls, ear weights were statistically significantly decreased in a dose-dependent manner. Additional histological indicators of inflammation were not observed.

Levan

An interleukin (IL)-1α release assay was used to determine the anti-inflammation effect of a 5% aq. levan solution on artificial skin.[57] Primary skin irritation was first induced using sodium lauryl sulfate (SLS). A dose of 0.01 or 0.05 mg/ml of the solution was applied to the skin. Levan decreased IL-1α release, indicating an anti-inflammatory effect.

Cytotoxicity

Levan

The cytotoxic effect of 5% levan (w/w) was determined using human fibroblasts and keratinocytes.[57] The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure cell viability and proliferation after 24 h incubation with levan. Levan, ≤100 µg/ml, was not cytotoxic to human fibroblasts. Levan had a proliferative effect in keratinocytes; proliferation was >30% at concentrations of >1 mg/ml.

Repeated Dose Toxicity

Repeated dose toxicity studies are summarized in Table 9. The oral toxicity of xanthan gum was evaluated in rats and dogs, of gellan gum in rats, dogs, and monkeys, of dextran in rats, of beta-glucan in mice, rats, and dogs, and of pullulan in rats. Most of the studies were dietary, and study durations lasted up to 2 yrs. Most observations were related to changes in feed consumption and intestinal effects. In guinea pigs, inhalation exposure to 100 µg/ml beta-glucan (as curdlan), 4 h/day, 5 days/wk for 4 wks, did not have an effect on the cells of the lung lavage or cell wall, and there were no microscopic lesions of the lung. With i.p. administration, no toxicity was reported when mice were dose 10 times over 2 wks with 5 mg xanthan gum in 0.5 ml water or mice or guinea pigs were dosed with 250 mg/kg bw beta-glucan for 7 days. Intravenous administration of 40 and 1000 mg/kg bw beta-glucan for 30 days resulted in hepatosplenomegaly in mice.

Oral Intake by Humans

Xanthan Gum

Five male subjects consumed 150 mg/kg bw/day xanthan gum as three measured portions daily for 23 days.[58] Ingestion of xanthan gum had no significant adverse effect on hematology, clinical chemistry, or urinalysis parameters.

Gellan Gum

Five male and five female subjects consumed a daily dose of 175 mg/kg gellan gum for 7 days and 200 mg/kg/day for the next 16 days.[58] No adverse dietary or physiological effects and no allergenic effects were reported.

Dextran and Pullulan

No adverse effects were seen in a study in which 10 g pullulan, dextran, and soluble starch were ingested by 8 male volunteers.[42,59] Each ingredient was administered for 14 days, and there was a 14-day wash-out period between treatments.

Beta-Glucan

Six male subjects ingested milkshakes with or without beta-glucan (as curdlan) for 28 days.[42] The test subjects were given 6 g/day for 5 days, 35 g/day for 2 wks, and 50 g/day from day 21-28. No evidence of toxicity was observed.

Pullulan

In a tolerance study, 13 subjects ingested 10 g/day of pullulan (50,000 MW) for 14 days.[44,46] There were no effects on clinical chemistry parameters, and no adverse effects were reported.

Industrial Exposure

Xanthan Gum

The relationship between the handling of xanthan gum powder and adverse symptoms was examined using exposure groups based on average percentage of time spent in a plant that uses a fermentation process to manufacture xanthan gum, not on the basis of expected intensity of exposure to xanthan gum.[60] The exposure of interest was to the employees exposed to milling, blending, and packaging of the product; a total of 39 employees were surveyed. Analysis of the results found no significant acute or chronic effects in pulmonary function in any of the exposure groups.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Oral

Xanthan Gum

A three-generation reproductive toxicity study was performed in which albino rats were fed dietary levels of 0, 0.25, and 0.5 g/kg bw/day xanthan gum.[61] Ten males and 20 females were used for the first generation, and 20 males and 20 females were used in the next two generations. Rats were mated to produce two litters per generation, and the successive generations were selected from weanlings of the second litter. Survival and reproductive parameters were similar for treated and control parental rats. Body weights of treated parental rats were slightly decreased compared to controls in each generation. There were no significant differences in developmental parameters between test and control litters, and no malformations were observed in any of the offspring.

Gellan Gum

Groups of 25 gravid female Sprague-Dawley rats were fed a diet containing 0, 2.5, 3.8, or 5.0% gellan gum (varied degree of acetylation; 58.5% polysaccharide) on days 6-15 of gestation.[39] No fetotoxic or teratogenic effects were reported. (No other details were provided.)

Beta-Glucan

A developmental study was performed with 0, 5, or 15% beta-glucan (as curdlan) with a control group of 40 male and 80 female CD rats and test groups of 20 male and 40 female rats.[42] The animals, which were mated twice, were fed the test diet throughout the study. Twenty of the treated dams nursed their own litters; the other 20 treated dams switched litters with the control dams so that treated animals would nurse control pups and control animals would nurse test pups. The F_{1a} offspring were killed prior to the second mating.

No changes in mortality, behavior, or appearance were observed. Male sires of the 15% beta-glucan group had decreased growth rates compared to controls, and males and females of the 15% group had decreased feed consumption. At birth, there were no differences in fertility or lactation among the groups, and no abnormalities were reported. However, survival of the F_{1a}, but not the F_{1b}, pups of the 5% group was statistically significantly decreased compared to controls. Weight gain of all F_{1a} litters of treated dams that nursed their own pups was statistically significantly decreased compared to controls; for the F_{1b} litters, the difference was statistically significant only in the 15% group. Statistically significant decreases in weight gain were also observed for pups of treated dams that were nursed by control dams, but the effect was reduced. Statistically significant decreased weight gain at some intervals was also observed for control pups nursed by treated dams. A no-observable effect level (NOEL) was not established.

The researchers also examined whether there would be decreased weight gain by the pups if dosing was discontinued during lactation. The protocol was similar to that just described, except that all groups consisted of 20 male and 40 female CD rats, and there was no cross-over at lactation. Weight gain by all pups during lactations was similar, although the researchers did state that the pups could have consumed parental diet from day 10+. The NOEL for parental toxicity and embryotoxicity was 15% beta-glucan.

A three-generation reproductive study was performed in which groups of 20 male and 40 female CD rats were fed a diet containing 0, 1, 5, or 15% beta-glucan (as curdlan) for 100 days.[42] The F_0 parents were mated twice, and the number of parents was halved after weaning of the first litter. The F_1 parents were mated three times and the F_2 parents were mated twice. The F_{1b} and F_{2b} litters were used to produce the next generation. After the third mating of the F_1 parents, half of the F_1 dams were killed on day 13 of gestation, and the remaining dams were killed on day 20 of gestation.

Mean growth and feed consumption were slightly decreased in male parental rats of the F_0 and F_1 generations of the 15% group. No gross or microscopic changes were observed in F_2 parents. No treatment-related effects on reproductive and developmental parameters were observed, but body weights of pups in almost all litters in all generations were statistically significantly decreased during lactation in the 15% group. Biologically-significant differences in body weights were not seen in litters of the other dose groups. No gross or microscopic lesions were observed in the F_{3b} pups of the 15% group. In the F_1 parents killed after the third mating, no reproductive or developmental effects were observed. Mean fetal weights in all groups were statistically significantly decreased compared to controls; however there was no dose-response. The NOEL for parental animals was 5%, based on decreased growth and increased cecal weights at 15% beta-glucan, and the NOEL for embryotoxicity was also 5%, based on decreased weight gain during lactation in the 15% beta-glucan group.

The teratogenic potential of beta-glucan (as curdlan) was determined using groups of 15-20 gravid Dutch belted rabbits.[42] The rabbits were dosed orally with 0, 1, 2, or 5 g/kg bw/day beta-glucan in a gelatin capsule delivered using a syringe. The 5 g/kg dose was administered as two divided doses, and the controls received two empty capsules. The rabbits were killed on day 28 of gestation. None of the controls died, but one, one, and three dams of the 1, 2, and 5 g/kg bw/day groups, respectively, died during the study. Eleven resorptions were observed in the high dose group, as compared to four in the control group, six in the 1 g/kg group, and five in the 2 g/kg group. The researchers stated, however, that the number of dams with resorptions was similar in all groups, and that no teratogenic effects were observed. The NOEL for both maternal and embryotoxicity was 5 g/kg bw/day.

GENOTOXICITY

Genotoxicity studies are summarized in Table 10. The in vitro genotoxicity of gellan gum (≤20 mg/ml), sodium dextran sulfate (≤25 mg/plate), beta-glucan (≤5000 µg/plate or /ml), sodium carboxymethyl beta-glucan (≤50,000 µg/plate or /ml) and pullulan (≤12 mg/ml) was evaluated using Ames test, chromosomal aberration assays, and/or DNA repair tests, with and without metabolic activation. The results were negative in these tests. The only non-negative result was a weak positive outcome with 20 mg/plate pullulan in a rec assay using *Bacillus subtilis*. Negative results were also reported in in vivo mouse micronucleus tests with ≤5000 mg/kg beta-glucan and ≤1800 mg/kg pullulan.

CARCINOGENICITY

Oral

Gellan Gum

Groups 50 male and 50 female Swiss Crl mice were fed a diet containing 0, 1, 2, or 3% gellan gum (varied degree of acetylation; 58.5% polysaccharide) for 96 wks (males) or 98 wks (females).[39] No treatment-related effects on body weights or feed consumption were observed. No treatment-related neoplastic or non-neoplastic lesions were reported.

In another study, groups of 50 male and 50 female F_1 generation Sprague-Dawley rats were exposed *in utero* to gellan gum, and were maintained on a diet containing 0, 2.5, 3.8, or 5.0% gellan gum (varied degree of acetylation; 58.5% polysaccharide) for 104 wks.[39] Survival of treated male rats was decreased when compared to controls, but survival of treated female rats was better than the concurrent controls. Male rats of the 3.8 and 5.0% test groups had decreased body weights compared to controls initially and after 76 wks. However, the researchers stated that the growth pattern of these test animals was the same as that of the controls, and the lower body weights were not indicative of toxicity. There were no neoplastic or non-neoplastic lesions associated with dosing, and gellan gum was not carcinogenic when fed to Sprague-Dawley rats.

Dextran

A group of 15 male and 15 female ACI rats were fed a diet containing 2.5% dextran (21,500 MW) for 480 days, and the control group of 20 males and 20 females was fed a basal diet.[62] Body weights gains of treated male rats were statistically significantly decreased compared to controls. An increased incidence in tumors was not reported, and no intestinal tumors were found.

Sodium Dextran Sulfate

A number of studies have demonstrated that oral exposure to sodium dextran sulfate produces colon cancer in rats; the mechanism is not genotoxic. Oral administration has been shown to induce colonic inflammation, and a 2-day study in which female Fischer 344 rats were given 3 or 6% sodium dextran sulfate in the drinking water indicated that oxidative DNA damage occurred in the colonic mucosa.[63] The MW of sodium dextran sulfate has been found to be a factor in carcinogenic activity. While an extensive number of studies are available in the published literature, just a few are summarized below. An example of an inflammation-related mouse colon carcinogenesis model is described, with an indication of strain-dependency.

In one study evaluating the carcinogenic potential of sodium dextran sulfate (54,000 avg. MW; sulfur content 18.9%) in ACI rats, 10 males were fed a diet containing 10% sodium dextran sulfate, 14 males and 12 females were fed 5% in the diet, and a control group of nine males and nine females were given a basal diet.[64] All animals were necropsied at natural death or when killed due to moribund condition. All animals in the 10% group died 6-14 days after initiation of dosing, and all had severe acute nephrosis. Two animals of the 5% group died on day 14, but most of the remainder of this group lived for more than 130 days. Blood was observed on the surface of the stools of these animals at 2.5 mos. The weight gain of animals of this group was decreased compared to controls. Of the 23 rats that survived for more than 130 days, 15 rats developed intestinal tumors; tumors included five adenomas, five adenocarcinomas, and three papillomas in the colon and six adenomas and two adenocarcinomas in the cecum. While most rats had a single tumor upon gross observation, microscopic examination found multicentric foci of atypical hyperplasia of the glandular epithelium. No intestinal tumors were reported in the control group.

In a second study testing the same sodium dextran sulfate, 15 male and 15 female ACI rats were fed 1% in the diet for 660 days; the avg. daily intake was 0.15 g/day/animal.[65] A control group of 10 males and 10 females were fed a basal diet. All but two treated male rats survived for 350+ days. Body weight gains of the test group were similar to that of the controls. Intestinal tumors were observed in 22 treated rats; 16 papillomas, four squamous cell carcinomas, two adenomas, and five adenocarcinomas were reported in the colon and rectum and one adenocarcinoma was found in the cecum. Thirteen tumors were reported at miscellaneous sites. Again, no intestinal tumors were reported in the control group.

To examine the effect of MW, three groups of 15 male and 15-16 female ACI rats were fed for 480 days a diet containing 2.5% of a sodium dextran sulfate, MW 520,000, 54,000,or 9500; each sodium dextran sulfate had a sulfur content of 18-19%.[62] (The 54,000 MW substance was synthesized using the dextran described previously.) A control group of 20 male and 20 female rats was fed a basal diet. There was no significant difference in survival time between any of the groups. Body weights gains of male rats of the 54,000 MW diet were statistically significantly decreased compared to control males; body weight gains in the other two groups were similar to controls. The 54,000 MW sodium dextran sulfate had the strongest carcinogenic activity, with tumors

being reported similar to the studies described previously. The other two MW substances did not show significant carcinogenic activity; only 2 colorectal adenocarcinomas were observed in the 520,000 MW group. Colorectal squamous metaplasia was observed in most rats in all test groups. Other miscellaneous tumors were reported, but there was no statistically significant difference between treated and control groups.

A colitis-related mouse colon carcinogenesis model was developed.[66] When eight male Crj:CD-1 mice were given a single i.p. injection of 10 mg/kg azoxymethane (AOM), followed by 7 days of 2% sodium dextran sulfate in the drinking water 1 wk later, there was a 100% incidence of colonic adenocarcinomas and 38% of adenomas at wk 20. No adenocarcinomas were observed in any of the mice dosed with AOM and sodium dextran sulfate simultaneously, 1 wk of sodium dextran sulfate and then AOM, AOM only, or sodium dextran sulfate only, and only two adenomas (in 10 mice) were observed when AOM was given during sodium dextran sulfate administration. All of the adenocarcinomas were positive for β-catenin, cyclooxygenase (COX)-2, and inducible nitric oxide synthase (iNOS) and negative for immunoreactivity of p53.

Strain-sensitivity to the above model was then examined.[67] In testing with male Balb/c, C3H/HeN, C57BL/6N, and DBA/2N mice, Balb/c mice were very sensitive to the model. C57BL/6N mice also developed colonic adenocarcinomas, but to a lesser extent. C3H/HeN and DBA/2N mice developed only a few colonic adenomas. However, the greatest inflammation response was observed in C3H/HeN mice, followed by Balb/c mice. The researchers hypothesized that the strain differences in susceptibility of colon carcinogenesis induced by AOM and sodium dextran sulfate might be influenced by the response to nitrosation stress due to inflammation as determined by the genetic background.

Anti-Tumor Effects

Xanthan Gum

Groups of C57BL/6 mice were inoculated subcutaneously (s.c.) with 1 x 10^6 $B16K^b$ melanoma cells.[68] A suspension of 10 mg/ml xanthan gum, 100 µl, or PBS was given by gavage once every 5 days, starting 1 day prior to inoculation. Rapid tumor growth occurred in PBS-treated mice, but tumor growth was statistically significantly reduced in xanthan gum-treated mice. Spleen cell composition was analyzed 22 days after inoculation. There was no change in overall cellular composition, but natural killer (NK) cells and tumor-specific cytotoxic T-lymphocyte (CTL) activity were increased by xanthan gum. All PBS-treated mice died by day 46 after inoculation; 40% of the xanthan gum-treated mice were alive at day 100. The researchers demonstrated that the anti-tumor effect of xanthan gum was highly dependent on Toll-like receptor (TLR) 4-mediated signaling.

Pullulan

Male Balb/c mice were used to determine the anti-tumor and anti-metastatic potential of pullulan (water-soluble low-MW β-(1→3)- with 50-80% branched β-(1→6); MW 100,000).[69] Colon-26 cells were implanted into the spleens of mice on day 0, and groups of mice were dosed orally with 25 or 50 mg/kg or i.p. with 5 or 15 mg/kg pullulan for 14 consecutive days, starting 12 h after implantation. Control groups consisted of sham-operated mice (not implanted with colon-26 cells) and mice implanted with the cells but given physiological saline or distilled water instead of pullulan. Splenic tumor weights were reduced with the 50 mg/kg oral and 15 mg/kg i.p. doses of pullulan, but not with the other doses. Liver metastasis was significantly inhibited with 50 (but not 25) mg/kg oral and 5 and 15 mg/kg i.p. pullulan.

Levan

The anti-tumor activity of levan produced from four different microorganisms, i.e., *Gluconoacetobacter xylinus*, *Rahnella aquatilis*, *Zymomonas mobilis*, and *Microbacterium laevaniformans* (G-levan, R-levan, Z-levan, and M-levan, respectively) was evaluated using female ICR mice.[70] The MWs of these levans were 40,000, 380,000, 570,000, and 710,000, respectively. On day 0, 0.2 ml (equiv. to 3 x 10^6 cells) of sarcoma-180 tumor cells were implanted s.c. into the groin of the mice. On days 1through 7, 200 mg/kg of the levans in distilled water were applied to the mice, and the mice were killed on day 26. The tumor growth inhibition ratio (IR) ranged from 42.2-69.6% for the four levans. M-Levan had the highest I.R. value, but it was not statistically significant. The IRs for R- and Z-levan were comparable, and the G-levan was significantly less effective.

IRRITATION AND SENSITIZATION

Non-human and human dermal irritation and sensitization studies are summarized in Table 11. The dermal irritation and sensitization potentials of xanthan gum, beta-glucan, and sodium carboxymethyl beta-glucan were evaluated in animal studies. Xanthan gum, up to 1%, and beta-glucan, concentration not specified, were not irritating to rabbit skin. One study with 5% aq. xanthan gum on shaved rabbit skin produced localized irritation; study details were not provided. Neither xanthan gum, tested at 0.1%, nor beta-glucan (concentration not specified) were sensitizers in guinea pigs. Sodium carboxymethyl beta-glucan was at most a slight skin irritant in guinea pigs at a concentration of 50% aq.; a 10% aq. solution was not a sensitizer in guinea pigs.

In humans, neither beta-glucan nor sodium carboxymethyl beta-glucan were irritants or sensitizers. The test concentration of beta-glucan was not specified. Sodium carboxymethyl beta-glucan was applied neat in the irritation study and as a 2% aq. solution in the sensitization study.

Phototoxicity

A human photoallergenicity study also is summarized in Table 11. A 2% aq. solution of sodium carboxymethyl beta-glucan was not photosensitizing in clinical studies.

Adverse Reactions

Dextran

Over a 5-yr period (1981-1986), 12,646 dextran 70 units were administered to 5745 patients (mean of 2.2 units/patient) undergoing gynecological surgery or a Cesarean section as a plasma volume expander.[71] Fifteen immediate dextran-induced anaphylactoid reactions were reported, with an incidence of one reaction/383 patients treated. Life-threatening reactions occurred in 7 of these patients.

Ocular Irritation

Ocular irritation studies are summarized in Table 12. Xanthan gum, 1%, and up to 0.8% gellan gum were not ocular irritants in rabbit eyes, and up to 0.5% gellan gum was not irritating to human eyes. The ocular irritation potential of sodium carboxymethyl beta-glucan was described as weakly irritating in a HET-CAM assay, but was practically non-irritating in rabbit eyes.

SUMMARY

Microbial polysaccharide gums can be produced intercellularly, by the cell wall, or exocellularly. Exocellular polysaccharide gums constantly diffuse into the cell culture medium and are easily isolated, while cell wall and intercellular polysaccharide gums are more difficult to separate from cell biomass. Many of the 34 microbial polysaccharide gums discussed in this safety assessment are produced exocellularly. They are reported to have numerous functions in cosmetics, including emulsion stabilizer, film former, binder, viscosity increasing agent, and skin conditioning agent.

The same microbial polysaccharide gums can often be produced by more than one organism. For example, beta-glucan can be produced by fungi, yeasts, and grains and levan can be produced by bacteria, yeasts, or fungi. The properties of the microbial polysaccharide gums can vary widely based on, among other parameters, the side groups, the ester substituents, or bacterial strains. These polysaccharide gums are generally very large molecules, and the MW of each ingredient can vary considerably.

Xanthan gum is reported to be used in almost every category of cosmetic product, with 3470 reported uses. Biosaccharide gum-1, sclerotium gum, and beta-glucan are reported to be used in 346, 193, and 137 cosmetic formulations, respectively. All other in-use ingredients have less than 70 uses. The ingredient with the highest concentration of use is pullulan; it is used at up to 12% in leave-on formulations and 17% in a breath freshener. Both xanthan gum and biosaccharide gum-1 are used at up to 6% in leave-on formulations and xanthan gum crosspolymer and biosaccharide gum-4 are used at 5% in leave-on formulations. All other in-use ingredients are used at concentrations of ≤3%.

Xanthan gum, gellan gum, and beta-glucan are approved as direct food additives, and xanthan gum and dextran are approved indirect food additives. Xanthan gum and dextran also have pharmaceutical applications.

Xanthan gum, orally administered, is slowly broken down in the gut by enzymatic and non-enzymatic mechanisms, and can be absorbed in some form to some extent. The absorbed fraction does not accumulate in the tissues and can be completely metabolized to CO_2. Gellan gum, orally administered, does not breakdown to any substantial extent in the gut, and is only very poorly absorbed.

Dextrans, 3,000-20,000 MW, are not absorbed through intact skin in humans. They are absorbed through the dermis (up to 38% for 3000 MW) if the epidermis is removed (e.g., via mini-erosion); absorption in this case is inversely proportional to MW. Orally administered dextrans, 4400 to 40,500 MW, are not absorbed to any appreciable extent in the gut (very low bioavailability). If dextrans were absorbed to a significant extent through the skin, animal studies in which dextrans were administered i.v. indicate that their half-lives in blood plasma would be directly related to the MW; they would be excreted in the urine to an extent that is inversely related to the MW; the lower MW dextrans would tend to be readily excreted unchanged in the urine; and the higher MW dextrans would have the potential to accumulate and to be broken down in the liver and other tissues, and would be more likely to be excreted in the urine in a dose-dependent manner than the lower MW dextrans. Dextran sulfate, 7000 to 8000 MW, orally administered, is only very poorly absorbed in the human GI tract. After i.v. administration, it is rapidly excreted, mostly intact, in the urine, although at least some of it can accumulate in the tissues (to a substantially greater extent than observed after oral exposure), and some of it appears to be incorporated into glycogen and other substances in the body.

Beta-glucan in a topically applied solution can penetrate into the epidermis and dermis. There appears to be no information about its absorption through the skin into the bloodstream. Orally administered, it is readily metabolized to CO_2, at least partially by microflora in the gut. Beta-glucan is not well absorbed or eliminated after i.p. injection. Pullulan, orally administered, is hydrolyzed to some small extent in the gut. It is partially hydrolyzed by amylases in the upper GI tract of human subjects, and is subject to breakdown by intestinal microflora to form short-chain fatty acids; the rate of the latter depends on the degree of polymeriza-

tion. However, pullulan can be essentially completely broken down to short-chain fatty acids in the human gut. It has not been determined to what extent, if any, the products of hydrolysis can be absorbed.

The acute toxicity of xanthan gum, gellan gum, beta-glucan, sodium carboxymethyl beta-glucan, and pullulan was assessed orally in mice, rats, and/or dogs, and dextran sulfate and beta-glucan were tested by i.p. and i.v. dosing in mice and rats. There was no notable toxicity observed in these studies. In acute inhalation studies, the LC_{50} of xanthan gum was >21 mg/l in rabbits and of gellan gum was >5.06 mg/l in rats. A single 180-min exposure of humans office dust contaiing 10 mg curdlin/g dust resulted in decreased nasal volume, in swelling in the nasal turbinates, and an increase in nasal eosinophils when compared to "clean" dust.

The inflammatory response following a single exposure to beta-glucan (as curdlan) and to pullulan was also evaluated in guinea pigs. Mostly, no effect or a slight decrease in inflammatory cells in lung lavage was observed. A 4 h inhalation exposure to beta-glucan in dust by guinea pigs produced a delayed subacute nasal congestion when compared to dust without beta-glucan and resulted in decreased nasal volume. Industrial exposure to xanthan gum powder did not appear to cause significant acute or chronic pulmonary effects. Using artificial skin, 5% levan had an anti-inflammatory effect in irritated skin.

Repeated dose oral toxicity of xanthan gum was evaluated in rats and dogs, of gellan gum in rats, dogs, and monkeys, of dextran in rats, of beta-glucan in mice, rats, and dogs, and of pullulan in rats. Most of the studies were dietary, and study durations lasted up to 2 yrs. Most observations were related to changes in feed consumption and intestinal effects. Inhalation exposure to 100 µg/ml beta-glucan (as curdlan), 4 h/day, 5 days/wk for 4 wks, did not have an effect on the cells of the lung lavage or cell wall, and there were no microscopic lesions of the lung. With i.p. administration, no toxicity was reported when mice were dose 10 times over 2 wks with 5 mg xanthan gum in 0.5 ml water or mice or guinea pigs were dosed with 250 mg/kg bw beta-glucan for 7 days. Intravenous administration of 40 and 1000 mg/kg bw beta-glucan for 30 days resulted in hepatosplenomegaly in mice.

No toxic effects were observed in human subjects with oral ingestion of 150 mg/kg/day xanthan gum or 175-200 mg/kg/day gellan gum for 23 days, 10 g of dextran or pullulan for 14 days, or 6-50 g/day beta-glucan for up to 28 days. No significant or chronic effects in pulmonary function were reported in groups exposed occupationally to xanthan gum.

Dietary reproductive and developmental toxicity studies were conducted with xanthan gum, gellan gum, and beta-glucan. No reproductive or developmental effects were reported in a three-generation reproductive study in which rats were fed diets containing up to 5 g/kg bw/day xanthan gum. Gellan gum, up to 5%, did not have a fetotoxic or teratogenic effect on rats. Dietary administration of beta-glucan in rats in reproductive and developmental studies did not have any reproductive effects, but there were statistically significant decreases in body weights and body weight gains in offspring and parental animals. In a teratogenicity study in which rabbits were dosed with 5 g/kg bw/day beta-glucan, an increase in resorptions in the 5 g/kg group was considered similar to the other test groups and the controls, and 5 g/kg beta-glucan was not teratogenic in rabbits.

The in vitro genotoxicity of gellan gum (≤20 mg/ml), sodium dextran sulfate (≤25 mg/plate), beta-glucan (≤5000 µg/plate or /ml), sodium carboxymethyl beta-glucan (≤50,000 µg/plate or /ml) and pullulan (≤12 mg/ml) was evaluated in Ames test, chromosomal aberration assays, and/or DNA repair tests, with and without metabolic activation. Results were negative in all of these tests. The only non-negative result was a weak positive result with 20 mg/plate pullulan in a rec assay using *Bacillus subtilis*. Negative results were also reported in in vivo mouse micronucleus tests with ≤5000 mg/kg beta-glucan and ≤1800 mg/kg pullulan.

Dietary studies examining the carcinogenic potential of ≤5% gellan gum and 2.5% dextran reported that neither of these ingredients caused an increase in tumors. However, a number of studies have demonstrated that oral exposure to sodium dextran sulfate produces colon carcinogenesis in rats, the mechanism is non-genotoxic. In one study, the MW of sodium dextran sulfate was a factor in carcinogenic activity; a 54,000 MW sodium dextran sulfate produced colorectal tumors, but 9500 and 520,000 MW sodium dextran sulfate did not have significant carcinogenic activity. Oral administration has been shown to induce colonic inflammation, and a 2-day study in which female Fischer 344 rats were given 3 or 6% sodium dextran sulfate in the drinking water indicated that oxidative DNA damage occurred in the colonic mucosa. An inflammation-related mouse colon carcinogenesis model indicated that the development of colonic tumors is strain-dependent, and that Balb/c mice were very sensitive to the model. C57BL/6N mice also developed tumors, but to a lesser extent, while C3H/HeN and DBA/2N mice only developed a few tumors.

Oral administration of 10 mg/ml xanthan gum had an anti-tumor effect in mice inoculated with melanoma cells, and 50 mg/kg pullulan, but not 15 mg/kg, significantly inhibited tumor growth in mice following implantation of colon-26 cells. Levan did not have an anti-tumor effect in mice.

Non-human and human dermal irritation and sensitization studies are summarized in Table 11. The dermal irritation and sensitization potentials of xanthan gum, beta-glucan, and sodium carboxymethyl beta-glucan were evaluated in animal studies. Xanthan gum, up to 1%, and beta-glucan, concentration not specified, were not irritating to rabbit skin. One study with 5% aq. xanthan gum on shaved rabbit skin produced localized irritation; however, study details were not provided. Neither xanthan gum, tested at 0.1%, nor beta-glucan (concentration not specified) were sensitizers in guinea pigs. Sodium carboxymethyl beta-glucan was at most a slight skin irritant in guinea pigs at a concentration of 50% aq.; a 10% aq. solution was not a sensitizer in guinea pigs.

In humans, neither beta-glucan nor sodium carboxymethyl beta-glucan were irritants or sensitizers. The test concentration of beta-glucan was not specified. Sodium carboxymethyl beta-glucan was applied neat in the irritation study and as a 2% aq. solution in the sensitization study. A 2% aq. solution of sodium carboxymethyl beta-glucan was not photosensitizing in clinical studies.

Xanthan gum, 1%, and gellan gum, up to 0.8%, were not ocular irritants in rabbit eyes, and up to 0.5% gellan gum was not irritating to human eyes. The ocular irritation potential of sodium carboxymethyl beta-glucan was described as weakly irritating in a HET-CAM assay, but the ingredient was practically non-irritating in rabbit eyes.

DISCUSSION

Microbial polysaccharide gums are produced by a wide variety of microorganisms, and some can also be isolated from plants, e.g., beta-glucan can be isolated from barley and oats. Although these ingredients are produced primarily by microbial sources, the cosmetic ingredients are purified during manufacture, thus microbial contamination is not a concern.

The Panel determined that, even though there are some data gaps, the available data on polysaccharide gums included in this safety assessment may be extrapolated to support the safety of the entire group. And while there was no specific data on the hydroxypropyltrimonium chloride compounds, data included in a previous CIR safety assessment on trimonium ingredients are applicable in determining the safety of the three hydroxypropyltrimonium chloride compounds included in this report.

Parenterally administered polysaccharides appear to be biotransformed to a limited but variable extent in animal and human studies. However, these very large compounds appear not to be significantly absorbed through the skin and would have negligible bioavailability. Coupled with a lack of significant toxicity associated with other routes of exposure, the CIR Expert Panel determined that systemic effects were unlikely to result from topical application of cosmetics containing these ingredients.

The Panel initially expressed concern for a study that reported 5% aq. xanthan gum caused irritation. However, this was the only finding of irritation among almost 20 studies on microbial polysaccharide gums. Given the absence of study details, including no mention of a control group, it appeared to the Panel to be more likely that the irritation was the result of the study methodology (e.g., shaved skin) and not by the xanthan gum. There was no evidence of sensitization in human or non-human testing.

The Panel also remarked on the induction of colon cancer in rodents with oral exposure to sodium dextran sulfate. Sodium dextran sulfate is a commonly used model for induction of colitis in a well-characterized mouse model to study colitis, and while the mode of action is unknown it is not relevant to human cosmetic exposure.

Finally, the Panel discussed the issue of incidental inhalation exposure to microbial polysaccharide gums from powders and other products that may be aerosolized. These ingredients are reportedly used at concentrations up to 1% in cosmetic products that may be aerosolized and up to 6% in other products that may become airborne. The Panel noted that 95% – 99% of droplets/particles would not be respirable to any appreciable amount. Furthermore, droplets/particles deposited in the nasopharyngeal or bronchial regions of the respiratory tract present no toxicological concerns based on the chemical and biological properties of this ingredient. Coupled with the small actual exposure in the breathing zone and the concentrations at which the ingredients are used, the available information indicates that incidental inhalation would not be a significant route of exposure that might lead to local respiratory or systemic effects. Results of acute inhalation studies with xanthan gum and gellan gum produced no significant toxicity, nor did results from a 4-wk inhalation study of beta-glucan in guinea pigs. Industrial exposure to xanthan gum powder caused no significant acute or chronic effects in pulmonary function. The Panel also considered the data available to characterize the potential for microbial polysaccharide gums to cause systemic toxicity, irritation, sensitization or other effects. The Panel noted that testing with a number of microbial polysaccharide gums demonstrated they did not produce systemic toxicity in oral studies; they are not reproductive or developmental toxicants; are not genotoxic; and are not considered irritants or sensitizers. A detailed discussion and summary of the Panel's approach to evaluating incidental inhalation exposures to ingredients in cosmetic products is available at http://www.cir-safety.org/cir-findings.

CONCLUSION

The CIR Expert Panel concluded the microbial polysaccharide gums listed below are safe in the present practices of use and concentration in cosmetics.

Xanthan Gum
Hydroxypropyl Xanthan Gum*
Undecylenoyl Xanthan Gum*
Dehydroxanthan Gum
Xanthan Gum Crosspolymer
Xanthan Hydroxypropyltrimonium Chloride*
Gellan Gum
Welan Gum*
Biosaccharide Gum-1
Biosaccharide Gum-2
Biosaccharide Gum-3*
Biosaccharide Gum-4
Biosaccharide Gum-5*
Pseudoalteromonas Exopolysaccharides*
Dextran
Carboxymethyl Dextran*
Dextran Hydroxypropyltrimonium Chloride*
Sodium Carboxymethyl Dextran
Dextran Sulfate
Sodium Dextran Sulfate

Sclerotium Gum
Hydrolyzed Sclerotium Gum
Beta-Glucan
Beta-Glucan Hydroxypropyltrimonium Chloride*
Beta-Glucan Palmitate*
Hydrolyzed Beta-Glucan*
Oxidized Beta-Glucan*
Sodium Carboxymethyl Beta-Glucan
Pullulan
Myristoyl Pullulan*
Levan*
Rhizobian Gum
Hydrolyzed Rhizobian Gum
Alcaligenes Polysaccharides

Were ingredients in this group not in current use (as indicated by *) to be used in the future, the expectation is that they would be used in product categories and at concentrations comparable to others in the group.

TABLES

Table 1. Definition, Function, and Idealized Structure

Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Xanthan Gum 11138-66-2	a high MW heteropolysaccharide gum produced by a pure-culture fermentation of a carbohydrate with *Xanthomonas campestris*;[14] composed of glucose, glucuronic acid, 6-acetylmannose, and 4,6-pyruvylated mannose residues[72]	binder; emulsion stabilizer; skin conditioning agent-misc.; surfactant-emulsifying agent; viscosity increasing agent-aq.	(given below definition)
Hydroxypropyl Xanthan Gum 106442-37-9	the hydroxypropyl ether of xanthan gum,[14] wherein ether substitution occurs primarily at 6-position of the backbone sugar residues	emulsion stabilizer; film former; viscosity increasing agent-aq.	

Table 1. Definition, Function, and Idealized Structure

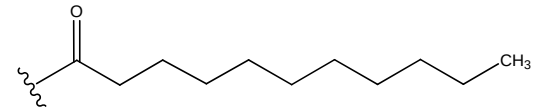
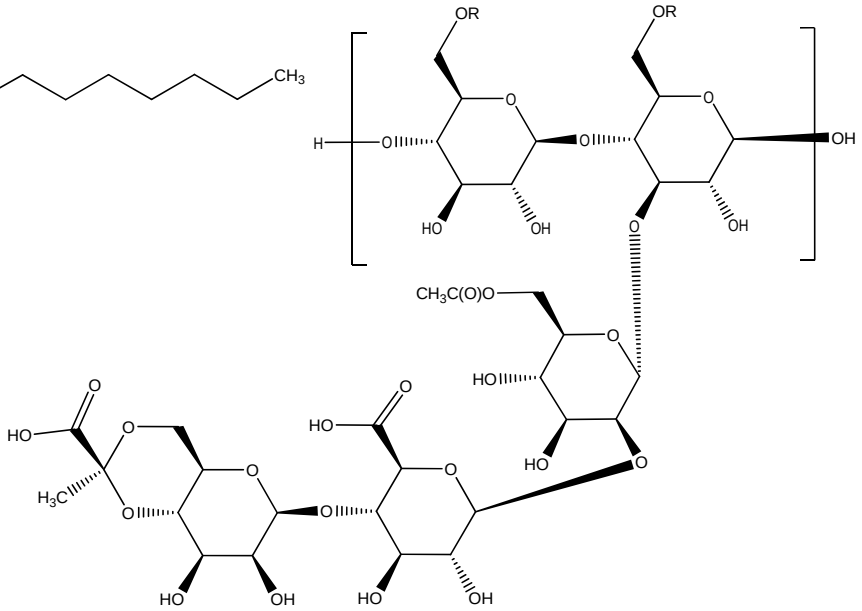
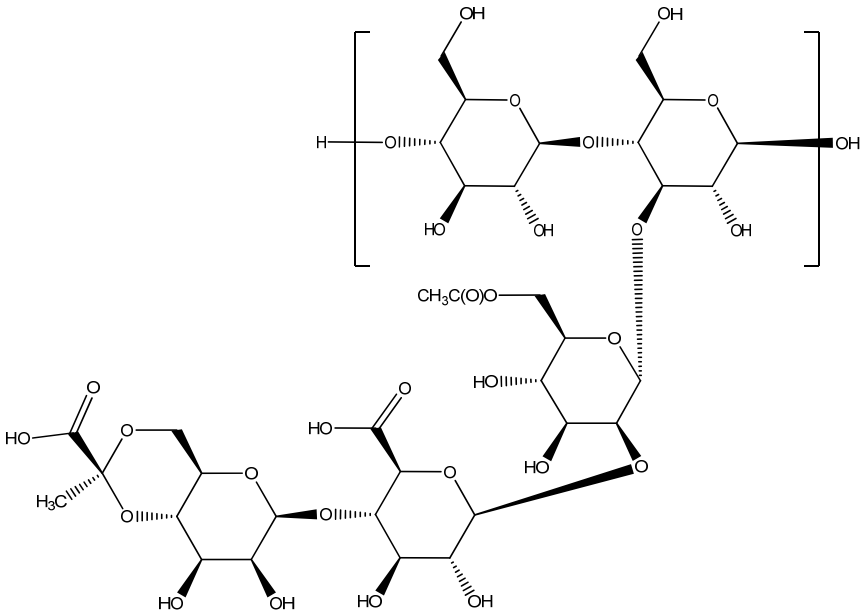
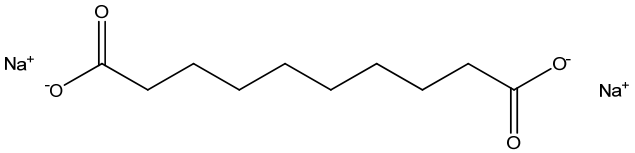
Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Undecylenoyl Xanthan Gum	the condensation product of undecylenic acid chloride and xanthan gum,[14] wherein esterification occurs primarily at the 6-position of backbone sugar residues	emulsion stabilizer; hair conditioning agent	wherein R = H or  
Dehydroxanthan Gum 11138-66-2 [dehydro is co-listed under this CAS No. with Xanthan Gum]	the product obtained by the dehydration of xanthan gum[14]	emulsion stabilizer; film former; hair fixative; suspending agent-nonsurfactant; viscosity increasing agent-aq.	
Xanthan Gum Crosspolymer	xanthan gum crosslinked with disodium sebacate[14]	skin protectant; skin conditioning agent-misc.	 Crosslinked with: 

Table 1. Definition, Function, and Idealized Structure

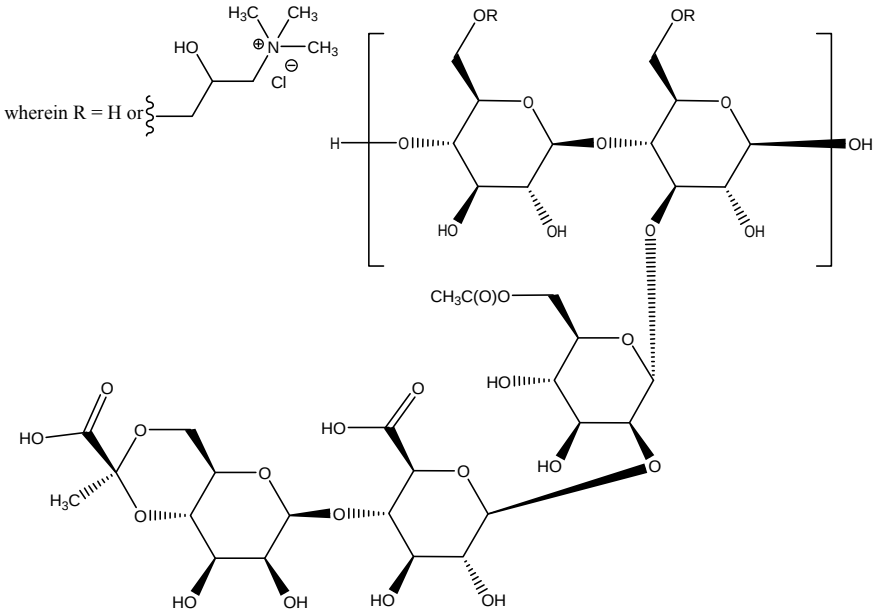
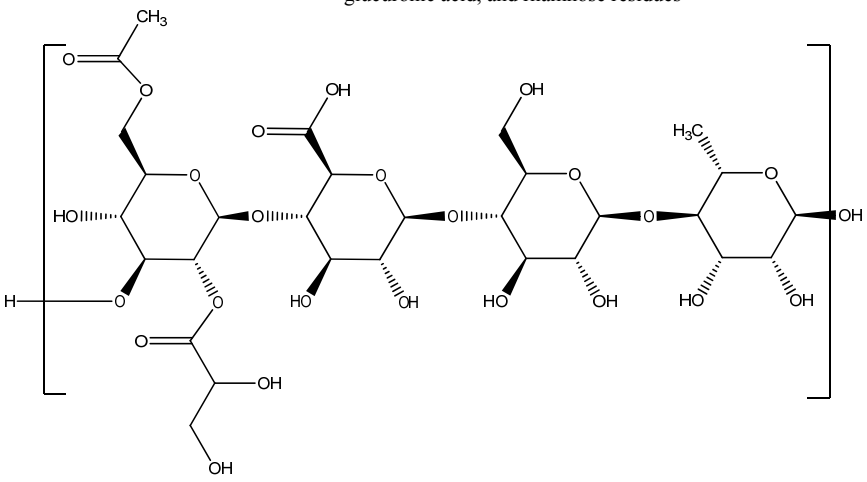
Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Xanthan Hydroxypropyl Trimonium Chloride	the quaternary ammonium salt formed by the reaction of Xanthan Gum with 2,3-epoxypropyltrimethylammonium chloride	hair conditioning agent; skin conditioning agent-humectant; viscosity increasing agent-aq	
Other Gums			
Gellan Gum 71010-52-1	a high MW heteropolysaccharide gum produced by pure-culture fermentation of a carbohydrate with *Pseudomonas elodea*;[14] consists of the basic backbone →3)-β-D-Glc-(1→4)-β-D-GlcA-(1→4)-β-D-Glc-(1→4)-α-L-Rha-(1→);[11] composed of glucose (some of which is glyceryl and/or acetyl esterified), glucuronic acid, and rhamnose residues[12]	emulsion stabilizer; viscosity increasing agent-aq.	

Table 1. Definition, Function, and Idealized Structure

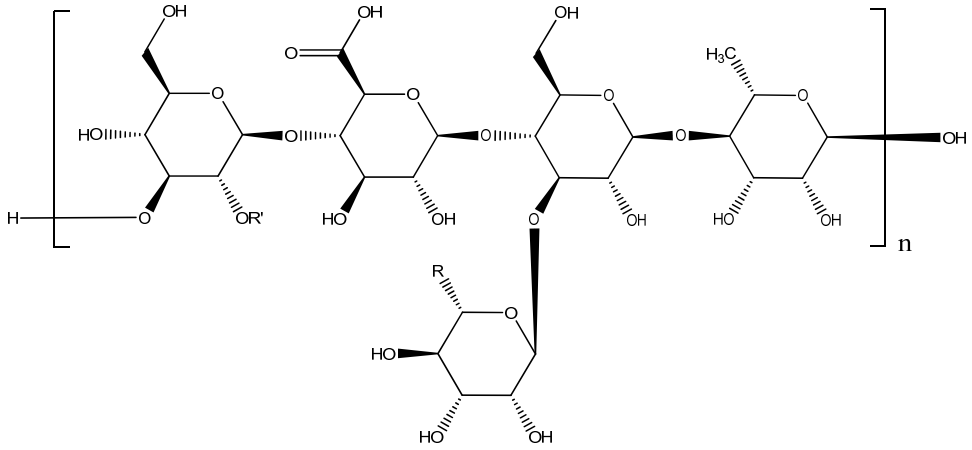
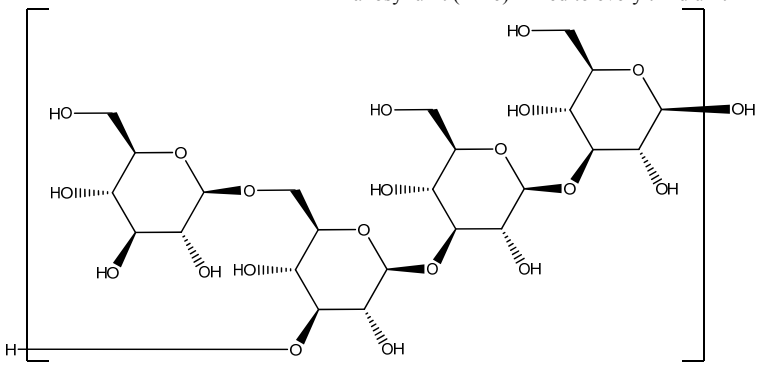
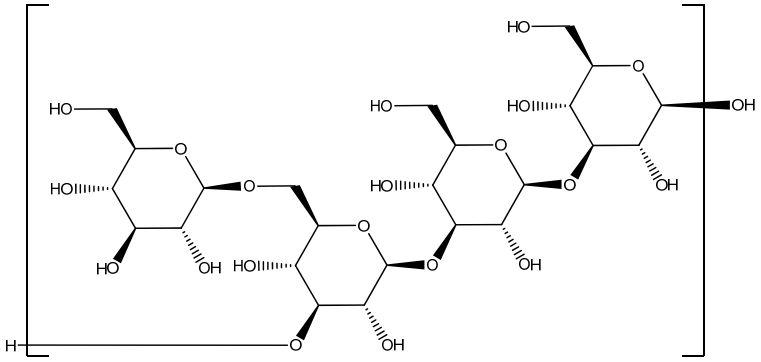
Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Welan Gum 96949-22-3	a gum produced by the fermentation of *Alcaligenes*;[14] consists of the basic backbone →3)-β-D-Glcp-(1→4)-β-D-GlcpA-(1→4)-β-D-Glcp-(1→4)-α-L-Rhap-(1→$^{3}\nwarrow_{1}$-α-L-Rhap **or** α-L-Manp; composed of glucose, glucuronic acid, rhamnose and mannose residues, with rhamnopyranosyl and/or L-mannopyranosyl side chains[73]	binder; emulsion stabilizer; film former; viscosity increasing agent-aq.	
wherein R is CH_3 or CH_2OH and R' is H or $C(O)CH_3$			
Sclerotium Gum 39464-87-4	the polysaccharide gum produced by the fungus, *Sclerotium rolfsii*;[14] consists of the basic backbone of β-D-(1→3)-glucopyranosyl units with a β-D-glucopyranosyl unit (1→6) linked to every third unit[74]	emulsion stabilizer; skin conditioning agent-misc.; viscosity increasing agent-aq.	
Hydrolyzed Sclerotium Gum	the partial hydrolysate of sclerotium gum derived by acid, enzyme, or other method of hydrolysis[14]	film former; skin protectant; skin conditioning agent-humectant	

Table 1. Definition, Function, and Idealized Structure

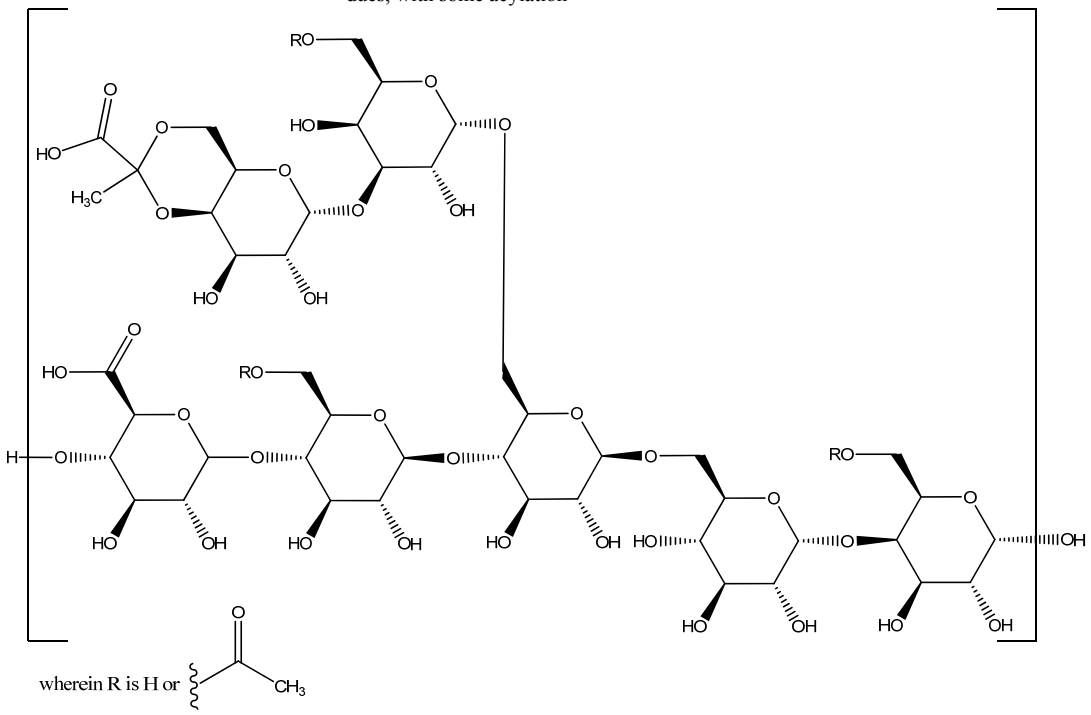
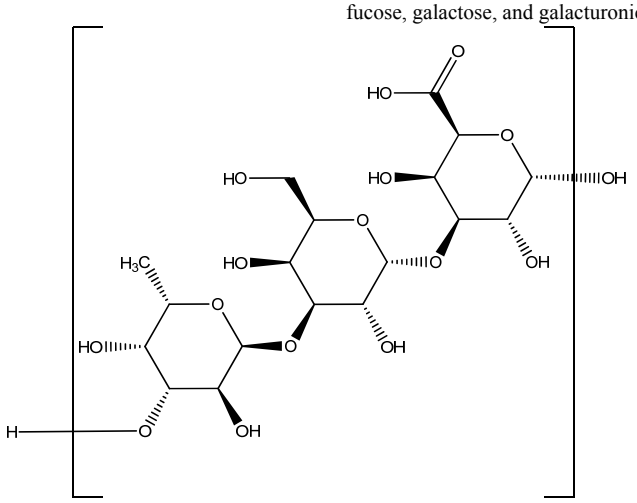
Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Rhizobian Gum 127712-52-1 [for Rhizob*ium* Gum]	the polysaccharide gum produced by the fermentation by *Rhizobium bacterium*;[14] composed of glucose, glucuronic acid, galactose and pyruvylated galactose residues, with some acylation[75]	film former; hair fixative; plasticizer; suspending agent-nonsurfactant; viscosity increasing agent-aq	
Hydrolyzed Rhizobian Gum	the partial hydrolysate of Rhizobian Gum derived by acid, enzyme, or other method of hydrolysis[14]	film former	
Biosaccharide Gum-1 223266-93-1	a fermentation gum derived from sorbitol; described as a polymer of α-L-fucose(1→3)α-D-galactose(1→3)-α-D-galacturonic acid trisaccharides; [14] composed of fucose, galactose, and galacturonic acid residues	skin conditioning agent-misc.	

Table 1. Definition, Function, and Idealized Structure

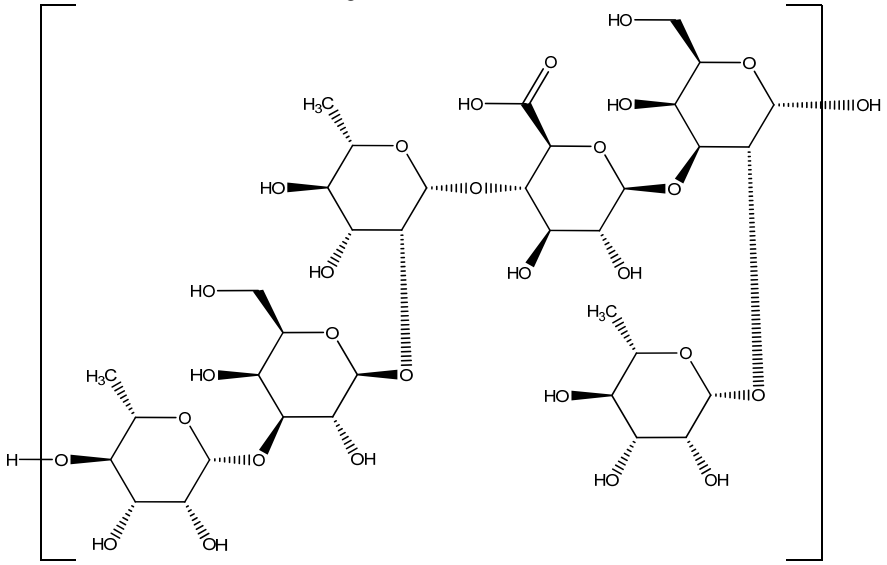
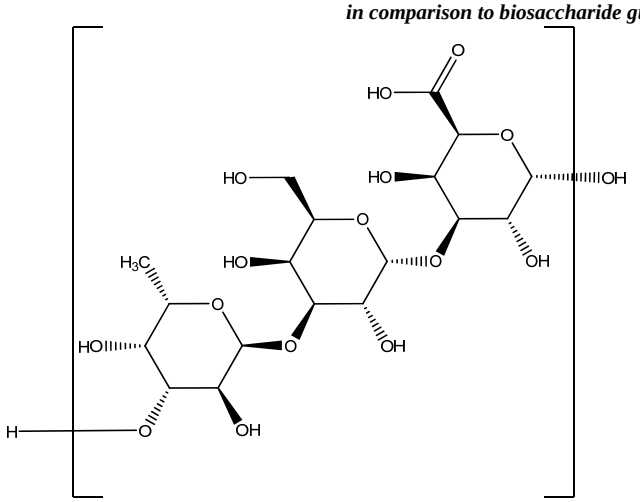
Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Biosaccharide Gum-2 758716-52-8	a fermentation gum derived from sorbitol; described as a polymer of α-L-Rhap(1→3)-β-D-Galp-(1→2)-α-L-Rhap-(1→4)-β-D-GlepA (1→3)-[α-L-Rhap-(1→2)-]-α-D-Galp-(1;[14] composed of rhamnose, galactose, and glucuronic acid residues	skin conditioning agent-misc.	
Biosaccharide Gum-3 896736-76-8	a fermentation gum derived from sorbitol; described as a polymer of α-L-fucose(1"3)-α-D-galactose(1"3)-α-D-galacturonic acid trisaccharides, ***and is characterized by a smaller degree of polymerization and MW in comparison to biosaccharide gum-1***[14]	skin conditioning agent-misc.	

Table 1. Definition, Function, and Idealized Structure

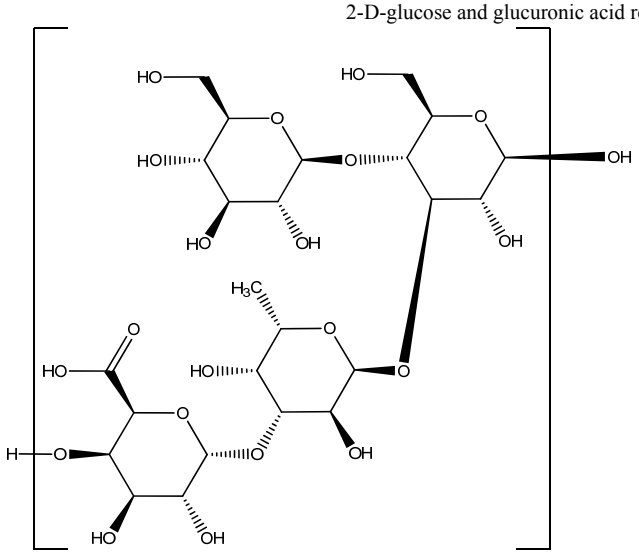
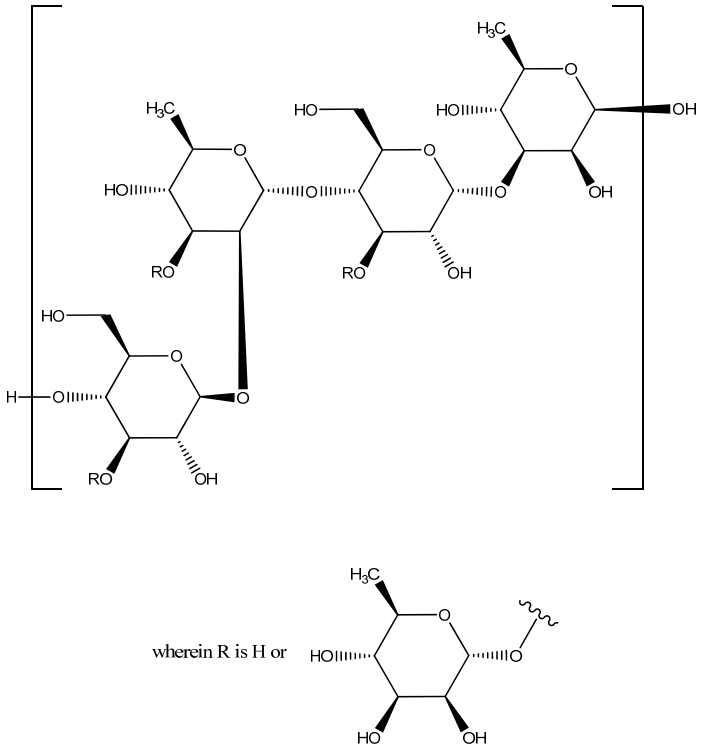
Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Biosaccharide Gum-4 283602-75-5	a fermentation gum derived from sorbitol; it is a de-acetylated branched polymer consisting of L-fucose, 2-D-glucose and glucuronic acid repetitive units[14]	skin conditioning agent-misc.	
Biosaccharide Gum-5	a fermentation gum derived from sorbitol; described as a polymer of –glucose-β-(1-->2)-rhamnose-α-(1-->4)-glucose-α-(1-->3)-rhamnose-β-(1-->4), with rhamnose –α-(1-->3) branching[14]	skin conditioning agent-misc.	
Pseudoalteromonas Exopolysaccharides	the exopolysaccharide excreted by the fermentation of *Pseudoalteromonas*	skin conditioning agent-misc.	

Table 1. Definition, Function, and Idealized Structure

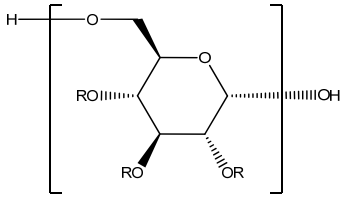
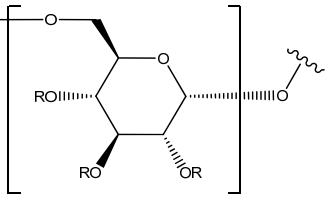
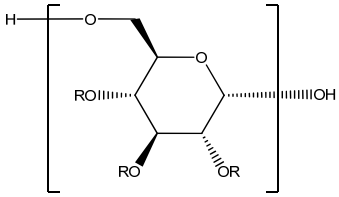
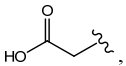
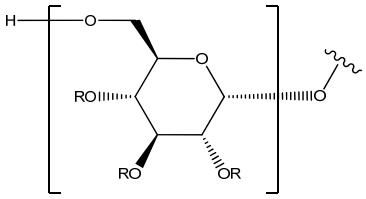
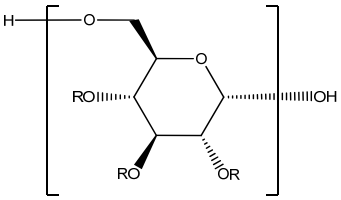
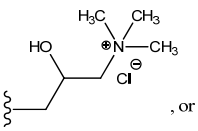
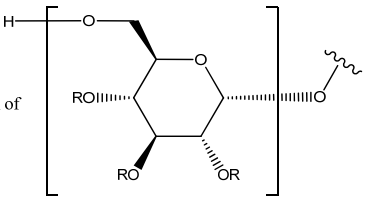
Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Dextran 9004-54-0	a high MW glucose polymer produced by the action of the bacteria, *Leuconostoc mesenteroides*, on a sucrose substrate[14]; the degree of branching is dependent on the source of dextrans and can vary from 0.5-60%[76]	binder; bulking agent	 wherein R = H or a branch of 
Carboxymethyl Dextran 9044-05-7	the carboxymethyl derivative of dextran[14]	film former; viscosity increasing agent-aq.	 wherein R = H,  , or a branch of 
Dextran Hydroxypropyltrimonium Chloride 83855-79-2	the quaternary ammonium compound formed by the reaction of dextran with glycidyltrimethylammonium chloride[14,77]	hair conditioning agent	 wherein R = H,  , or a branch of 

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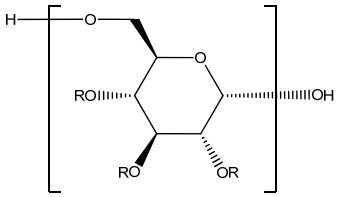
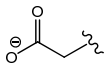
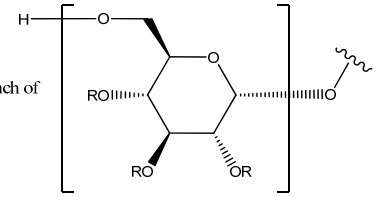
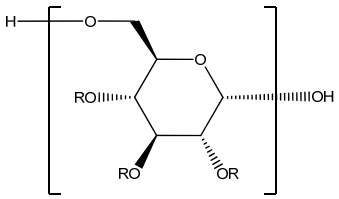
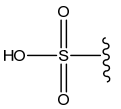
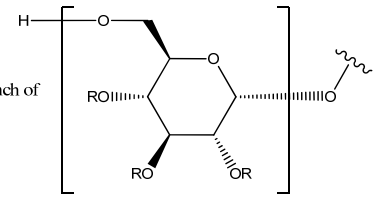
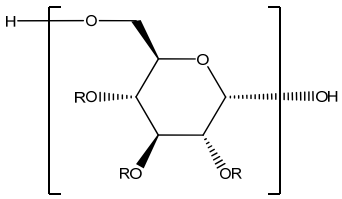
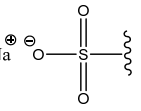
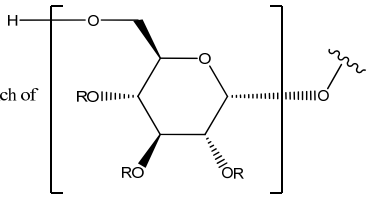
Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Sodium Carboxymethyl Dextran 39422-83-8	the sodium salt of a carboxymethyl dextran[14]	binder; emulsion stabilizer; viscosity increasing agent-aq.	 wherein R = H,  , or a branch of 
Dextran Sulfate 9042-14-2	the sulfuric acid ester of dextran[14]	binder; skin conditioning agent-misc.	 wherein R = H,  , or a branch of 
Sodium Dextran Sulfate 9011-18-1	the sodium salt of the sulfuric acid ester of dextran[14]	suspending agent-nonsurfactant	 wherein R = H,  , or a branch of 

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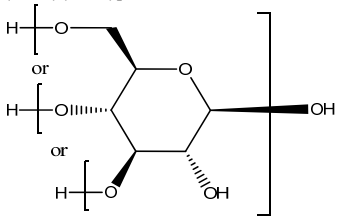
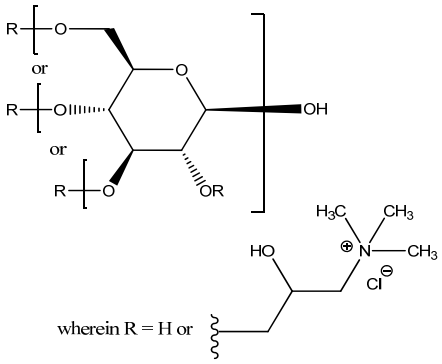
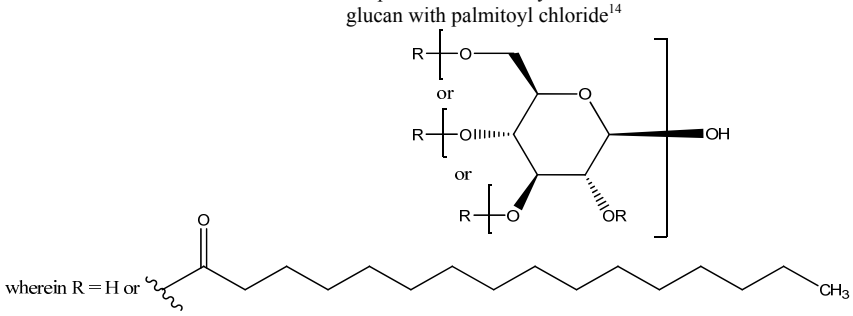
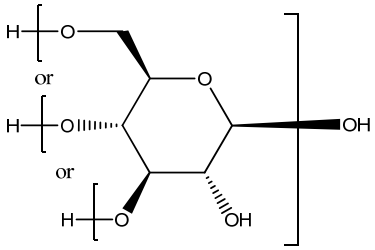
Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Beta-Glucan 55965-23-6 [CAS No. is specific to (1→3)(1→4)] 53238-80-5 [CAS No. is specific to (1→3)(1→6)] 	a polysaccharide consisting of β(1-3), β(1-4), and β(1-6) linked glucose units[14]	bulking agent; skin conditioning agent-misc	
Beta-Glucan Hydroxypropyl-trimonium Chloride 	the quaternary ammonium compound formed by the reaction of beta-glucan with glycidyltrimethylammonium chloride	anti-static agent; hair conditioning agent; skin conditioning agent - misc	
Beta-Glucan Palmitate 	the product obtained by the condensation of beta-glucan with palmitoyl chloride[14]	skin conditioning agent - misc	
Hydrolyzed Beta-Glucan 	the partial hydrolysate of beta-glucan, derived by acid, enzyme or other method of hydrolysis	skin conditioning agent - misc	

Table 1. Definition, Function, and Idealized Structure

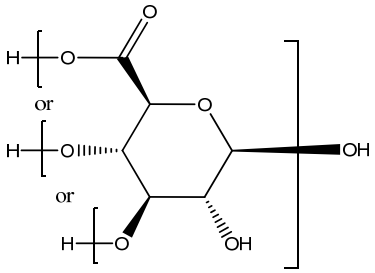
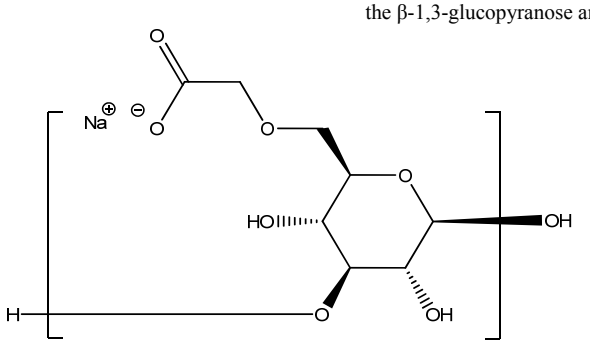
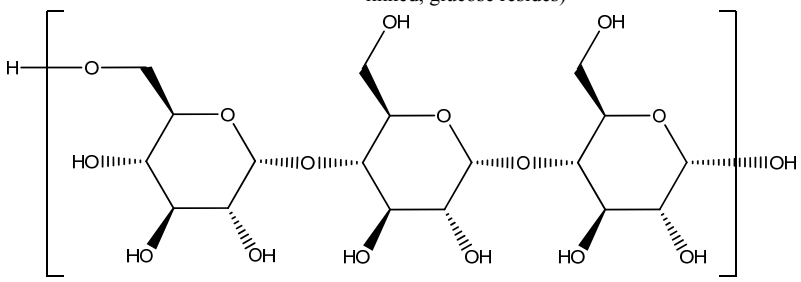
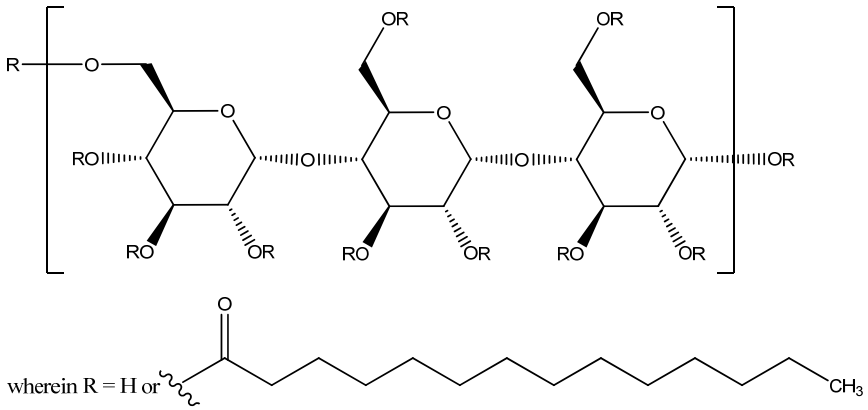
Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Oxidized Beta-Glucan	the product obtained by the oxidation of beta-glucan,[14] wherein the 6-position is oxidized to the acid[78]	anti-acne agent; anti-dandruff agent; anti-fungal agent; antimicrobial agent; deodorant agent; exfoliant; skin conditioning agent - misc	
Sodium Carboxymethyl Beta-Glucan 9050-93-5 [CAS No. specific to (1→3) β-D-Glucan]	the sodium salt of a carboxymethyl ether of beta-glucan, wherein the ether substitution occurs primarily at the 6-position[79]; 3 of 4 glucose-units of the β-1,3-glucopyranose are carboxymethylated[80]	binder; viscosity increasing agent - misc	
Pullulan 9057-02-7	an extracellular polysaccharide produced from starch by cultivating the yeast, *Aureobasidium pullulans*; composed of 1→6 linked maltitriose residues (a maltitriose is a block of three 1→4 linked, glucose resides)[81]	binder; film former	
Myristoyl Pullulan 1033464-89-9	the reaction product of myristoyl chloride and pullulan[14]	film former; hair fixative; hair-waving/straightening agent	

Table 1. Definition, Function, and Idealized Structure

Ingredient; CAS No.	Definition	Reported Function(s)[14]	Idealized Structure
Levan 9013-95-0	a polyfructose (fructofuranose), with some branching, produced by enzymatic action on sucrose[81]; the degree of branching varies by organism, but has been reported as high as 20%[82]	film former; skin protectant; viscosity increasing agent – aq.	
Alcaligenes Polysaccharides 188846-47-1 [for the pure disaccharide repeat unit]	the polysaccharides produced by a bacterial culture of *Alcaligenes latus*; composed of mannose and fucose[83]	emulsion stabilizer; humectant; skin conditioning agent – humectant; viscosity increasing agent-aqueous	

Table 2. Chemical and physical properties

Property	Description
	Xanthan Gum
appearance	cream-colored, odorless, free-flowing powder[35] white/beige powder with a characteristic odor[84]
MW	1,000,000 – 10,000,00 [33] varies within a very wide range[85]
solubility	dissolves readily in water with stirring to give highly viscous solutions at low concentrations[35] completely soluble in water, forming colloidal solution; insoluble in alcohol[84] readily soluble in hot or cold water to form neutral, viscous, and nonthixotropic solutions that have relatively high viscosity[33]
stability	resistant to heat degradation[35]
ionic nature	anionic[9,81]
pH	5.5-8.5 (1% solution, 25°C) [84]
	Gellan Gum
appearance	off-white powder[85-87]
types	native, deacetylated, clarified (i.e., filtered deacetylated gum)[86]
MW	varies within a very wide range[85] ~500,000[86] >70,000, with 95% above 500,000[39]
solubility	soluble in hot or cold deionized water[87] soluble in water; insoluble in ethanol[86]
viscosity	can exhibit high viscosity in solution[11] high-acyl gellan gum is viscous; deacylated gellan gum (treated with an alkali) has relatively low solution viscosity[13] cold dispersions of native gellan gum provide extremely high viscosities, and the solutions are highly thiotropic; the viscosity decreases with heating as the gum hydrates; hot native gum solutions are more viscous than deacylated gellan gum solutions[13]
gelling property	forms a weak gel in water in its native state, but forms a rigid gel after treatment with an alkali[12]
ionic nature	anionic[9,81]
hydration	native (acylated) gellan gum will swell in deionized water forming a very thick particulate system, and the hydration temperature is reached at ~70°C; the swelling behavior and hydration temperature is altered in the presence of ions[13] deacylated gum will only partially hydrate in cold deionized water, with hydration occurring with a heated deionized water temperature of ~70°C; also hydration is poor in mildly acidic conditions and in the presence of divalent ions[13]
	Welan Gum
MW	865,000-932,000[73]
viscosity	non-gelling polysaccharide forming highly viscous aq. solutions[88]
ionic nature	anionic[9]
	Biosaccharide Gum-1
MW	1,000,000 (avg)[89]
	Biosaccharide Gum-4
MW	2,000,000 (avg)[89]
	Dextran
appearance	Dextran 1: white to off-white powder[35]
MW	Dextran 1: ~1000 (avg) [90] Dextran 40: ~25,000 (solution has an avg MW of 40,000)[71] Dextran 70: ~40,000 (solution has an avg MW of 70,000)[71] MW distribution is dependent on the source of the dextran[76]
solubility	Dextran 1: very soluble in water; sparingly soluble in alcohol[35] degree of solubility decreases with an increase in the degree of branching; dextrans with >43% branching are insoluble[76]
ionic nature	nonionic[9,81]
stability	stable under mild acidic and basic conditions[76]
pH	Dextran 1: 4.5-7.0 (15% aq. solution) [90]
specific rotation	Dextran 1: between +148° and +164° at 20°, for an aq. solution[90] Dextran 40: between +195° and +203° [90] Dextran 70: between +195° and +203° [90]
	Dextran Sulfate
MW	5000-500,000 [10]

Table 2. Chemical and physical properties

Property	Description
	Sodium Dextran Sulfate
appearance	white powder[35]
MW	4000-50,000 [35]
solubility	freely soluble in water[35]
	Sclerotium Gum
solubility	disperses easily in water at room temperature; refined grades dissolve readily in hot and cold water[8]
ionic nature	nonionic[9,81]
	Beta-Glucan
appearance	white to pale yellow powder with a slight odor[91] white to nearly white powder (as curdlan)[87]
MW	~500,000 (native state)[92]
solubility	as curdlan: insoluble in water, alcohol, and most organic solvents; soluble in alkaline solutions[8]
ionic nature	nonionic[9]
	Oxidized Beta-Glucan
MW	continuum of ~30,000 to >70,000[92]
	Sodium Carboxymethyl Betaglucan
appearance	white/brown solid[93] amber, white granulate with a characteristic isopropyl alcoholic odor[80]
MW	~100,000[93] 4.23 x 10^5 (avg)[79]
degree of substitution	0.75 ±1[80,93] (as a 2% aq. solution) 0.2-0.3[79]
solubility	solubility up to 98%[79]
pH (2% aq. solution)	~7 [93]
	Pullulan
appearance	white to slightly yellowish powder; tasteless; odorless (food grade) [94] tasteless, odorless fine white powder[95]
MW	can vary considerably; a commercially available product has a molecular mass of 200,000 Da[31] 8000 - >2,000,000; approx. 200,000 (mean) [94]
solubility	highly soluble in cold or hot water; not soluble in organic solvents, except dimethylformamide or dimethyl sulfoxide (DMSO) [32,94]
stability	stable in aq. solution over a wide pH range; decomposes upon dry heating, carbonizing at 250-280°C [31]
viscosity	dissolves in water producing a stable viscous solution; does not gel; viscosity is proportional to MW[94] solutions are of relatively low viscosity; viscosity is stable to heating, changes in pH, and most metal ions[96]
ionic nature	nonionic[9,81]
pH	5.0-7.0 (food grade) [94]
refractive index	significant positive linear correlation of concentration and refractive index at 20 and 45°C [94]
	Levan
MW	up to several million Daltons; typically 2x10^6 to 10^8 [7] usually >0.5 million, and can be as high as 40,000,000 [82]
particle size	partially forms nanoparticles in water; 224.3 nm in water and 251.8 nm in ethanol[57]
solubility	highly soluble in water at room temperature[7] water soluble; does not swell in water[82]
viscosity	"exceptionally low" intrinsic viscosity[82]
ionic nature	nonionic[81]
	Rhizobian Gum
MW	1,500,000 (native molecule, in the fermentation broth)[75]
melting point	~60°C; gets lower after sterilization[97]
viscosity	with a 10 g/l solution, the viscosity decreases as the pH increases[75]
	Alcaligenes Polysaccharides
MW	64,000 [83]

Table 3. Constituents/Impurities

Ingredient	Constituents/Impurities
Xanthan Gum	nitrogenous constituents equal to approx.. 1% nitrogen by wt; approx.. half of the nitrogenous matter is proteinaceous and contains amino acid residues in the same proportions as other food-grade gums; the remainder is present as amino sugars, nucleic acids, and nucleotides[29]
	food-grade: contains D-glucose and D-mannose as the dominant hexose units, and D-glucuronic acid and pyruvic acid; NMT 2 mg/kg lead; NMT 0.075% ethanol and isopropyl alcohol, singly or combined[98]
	2.5-4.8% pyruvic acid, present as side chains[33]
Gellan Gum	usually contains a small amount of nitrogen-containing compounds as a result of the fermentation procedure[86]
	< 2 mg/kg lead; <3% nitrogen; ,750 mg/kg isopropyl alcohol[86]
	native: can contain 10% protein and 7% ash; deacetylated: can contain 17% protein and 8% ash[86]
	gellan gum is characterized by the polysaccharide content, percent of o-acetylated substitution, and protein content (including nucleic acid residues and other organic nitrogen sources)[39]
Dextran Sulfate	can contain polymers of various MWs and degrees of sulfation[41]
Beta-Glucan	≥85% β-1,3-1,6-glucan, sucrose, yeast extract, minerals, *Auerobasidium pullulans*[91]
	as curdlan: ≥90% carbohydrate; ≤10% water[8]
	as food grade curdlan – NMT 0.5 mg/kg lead; microbial limits, aerobic plate count NMT 1000 CFU/g; *Escherichia coli*, negative in 1 g[99]
Sodium Carboxymethyl Betaglucan	~90% sodium carboxymethyl betaglucan; <0.5% nitrogen (protein); <1.0% glycolic acid; <0.05% chloroacetic acid; ~10% volatile matter[80]
Pullulan	≥90% glucan on a dried basis; main impurities are mono-, di-, and oligosaccharides from the starting material[31]
	>90% pullulan; <10% mono-, di-,and oligosaccharides; <0.1 ppm lead; <2 ppm arsenic; <5 ppm heavy metals (food grade) [94]

Table 4. Methods of Manufacture/Organisms Used in Production

Ingredient	Method
Xanthan Gum	pure-culture fermentation of glucose or corn syrup from the bacterium *Xanthomonas campestris*; the polysaccharide is recovered by precipitation and purification with isopropyl alcohol, followed by drying and milling[33,60]
Gellan Gum	aerobic submerged fermentation using the bacterium *Pseudomonas elodea*; small seed fermentation is followed by pasteurization; the gum is recovered by precipitation with isopropyl alcohol, followed by drying and milling[100]
	pure culture fermentation of carbohydrates by *Pseudomonas elodea*, purified by recovery with isopropyl alcohol, dried, and milled[86]
	gellan gum can be recovered using alcohol precipitation (high acyl gum) or with alkali (deacylated gum)[13]
	produced by an organism that appears to belong to the *Auromonas* (ATCC 31461) genus; glycerate substitution predominates over acetate[11]
	Sphingomonas paucimobilis produce gellan gum[86]
	production is affected by media components, carbon source, nitrogen source, precursors, agitation rate, pH, temperature, and oxygen[86]
Welan Gum	fermentative production by *Alcaligenes* CGMCC2428 [73]
	produced by an *Alcaligenes* species (ATCC 31555)[11,88]
Dextran	Dextran 40; Dextran 70: obtained by the controlled hydrolysis and fractionation of polysaccharides elaborated by the fermentative action of certain appropriate strains of *Leuconostoc mesenteroides* on a sucrose substrate[90]
	the fermentation process for reaching high MW dextran takes place at 25°C; at lower temperatures, the amount of low MW dextran increases; at >25°C, higher branching occurs[10]
	different strains of the same bacterium produce dextrans with differing branched structures[71]
	the sucrose concentration also affects branching; increased sucrose content the degree of branching and the yield of high MW dextran decreases[10]
	dextran can be synthesized by dextrinase of different *Gluconobacter* species[10]
	dextran can be produced enzymatically using cell-free culture supernatants that contain dextransucranase[10]
	dextran can be synthesized chemically via a cationic ring-opening polymerization of levoglucosan[10]
Carboxymethyl Dextran	carboxymethylation of dextran in water/organic solvent mixtures using monochloroacetic acid under strong alkaline conditions[10]
Sclerotium Gum	produced by *Sclerotium glucanicum*[81] or *Sclerotium rolfsii*[8]
Beta-Glucan	extraction of extracellular β-glucan produced by the black yeast *Aureobasidium pullulans*[91]
	produced by fungi, yeasts, and grains (such as oat and barley); beta-glucans present in cereal bran are commonly produced as agricultural by-products[6]
	as curdlan: pure-culture fermentation of a carbohydrate by a nonpathogenic and nontoxigenic strain of *Agrobacterium biobar* 1 (formerly *Alcaligenes faecalis* var. myxogenes) or *Agrobacterium radiobacter*[99]

Table 4. Methods of Manufacture/Organisms Used in Production

Ingredient	Method
Oxidized Beta-Glucan	oxidation of beta-glucan is performed using phosphoric acid and sodium nitrite, with the actual oxidant being NO_2 gas; extent of oxidation was typically 10-20%[92]
Sodium Carboxymethyl Betaglucan	derived from the inner cell walls for baker's yeast (*Saccharomyces cerevisiae*); 3 of 4 glucose units of the β 1,3-glucopyranose are carboxymethylated[80,93]
	particulate glucan and sodium hydroxide are mixed, the sodium salt of monochloroacetic acid in 95% ethanol is added and stirred, excess sodium hydroxide is neutralized, the product is washed with 80% ethanol and dried[79]
Pullulan	fermentation of liquefied corn starch by *Aureobasidium pullulans*; the fungal biomass is removed by microfiltration, the filtrate is heat-sterilized, and the pigments and other impurities are removed by adsorption and ion-exchange chromatography[31]
	one company reports the following raw materials: ammonium sulfate; beer yeast extract; calcium hydroxide; caustic soda; corn syrup; diatomaceous earth; diammonium phosphate; dipotassium phosphate; hydrochloric acid; ion exchange resin; magnesium sulfate; salts; silicone oil; sodium glutamate; zinc carbon chloride[94]
	produced by *Pullularia pullulans* IFO 6353, *Dermatium pullulans* IFO 4464, etc in a culture medium containing a carbon source (such as glucose, fructose, etc) under anaerobic conditions[101]
Levan	produced extracellularly from sucrose- and raffinose-based substrates by levansucrase from a wide range of taxa, such as bacteria, yeasts, and fungi, but mainly by bacteria[7]
	fermentation of *Zymomonas mobilis* in a medium that contains 10% sucrose and 1% yeast extract, centrifugation via ultrafiltration, precipitation by the addition of ethanol, resuspension with distilled water, and drying[57]
	sources include Erwinia herbicola, Aerobacter lavanicum, Streptococcus salivarius, Pseudomonas prunicola, Arthrobacter acetigenum, Bacillus polymyxa, Bacilus subtilis, Actinomyces sp.[81]
Rhizobian Gum	produced by fermentation of Rhizobium sp. strain[75]
	bacterial strain YAS 34 is isolated from the rhizosphere of the sunflower plant; selection of the isolates is carried out on high carbon:nitrogen ratio liquid media; the culture broth was inoculated and fermented; the exopolysaccharide is recovered with a multi-step downstream processing that includes heating and centrifugation; diafiltration is used to eliminate fermentation residue, followed by further purification by alcoholic precipitation[102]
Alcaligenes Polysaccharides	neutral polysaccharide: culture broth was precipitated with ethanol and redissolved in hot water, filtration was used to remove the water-insoluble cells and acidic polymers, and the polysaccharide was recovered by ethanol precipitation and further purified[83]

Table 5. Frequency and concentration of use according to duration and type of exposure

	# of Uses[15]	*Max. Concs. of Use (%)*[16]	*# of Uses*[15]	*Max. Concs. of Use (%)*[16]	*# of Uses*[15]	*Max. Concs. of Use (%)*[16]
	Xanthan Gum		**Dehydroxanthan Gum**		**Xanthan Gum Crosspolymer**	
Totals*	**3470**	**0.00001-6**	**15**	**0.1-1**	**2**	**0.03-5**
Duration of Use						
Leave-On	*2782*	*0.001-6*	*6*	*0.1-1*	*2*	*0.03-5*
Rinse-Off	*678*	*0.000001-6*	*9*	*0.4-0.8*	*NR*	*NR*
Diluted for (Bath) Use	*10*	*0.5-3*	*NR*	*NR*	*NR*	*NR*
Exposure Type						
Eye Area	292	0.001-2	1	NR	NR	NR
Incidental Ingestion	35	0.03-2	NR	NR	NR	NR
Incidental Inhalation-Spray	121[a]	0.2-1[a]; 0.05[b]	1[a]	0.1[a]-0.2	NR	NR
Incidental Inhalation-Powder	19	0.3-6	NR	NR	NR	NR
Dermal Contact	3179	0.001-6	12	0.1-0.8	2	0.03-5
Deodorant (underarm)	1[c]	0.005-0.6[c] not a spray: 0.4-1	NR	NR	NR	NR
Hair - Non-Coloring	129	0.000001-4	3	0.7-1	NR	NR
Hair-Coloring	59	0.2-6	NR	NR	NR	NR
Nail	11	0.2-3	NR	NR	NR	NR
Mucous Membrane	206	0.03-4	5	0.4	NR	NR
Baby Products	29	0.2-0.6	NR	NR	NR	NR

	Gellan Gum		**Biosaccharide Gum-1**		**Biosaccharide Gum-2**	
Totals*	**37**	**0.0004-0.5**	**346**	**0.002-6**	**14**	**1**
Duration of Use						
Leave-On	*35*	*0.0004-0.5*	*301*	*0.002-6*	*10*	*1*
Rinse Off	*2*	*NR*	*43*	*0.006-5*	*4*	*NR*
Diluted for (Bath) Use	*NR*	*NR*	*2*	*NR*	*NR*	*NR*
Exposure Type						
Eye Area	5	0.0004	28	0.01-1	2	NR
Incidental Ingestion	1	0.0004	NR	0.08	NR	NR
Incidental Inhalation-Spray	NR	NR	3[a]	0.002[a]	NR	NR
Incidental Inhalation-Powder	6	0.0004	1	NR	NR	NR
Dermal Contact	34	0.0004-0.3	326	0.002-6	14	1
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	1	0.5	19	NR	NR	NR
Hair-Coloring	NR	NR	1	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	2	0.0004	4	0.08	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR

	Biosaccharide Gum-4		**Dextran**		**Sodium Carboxymethyl Dextran**	
Totals*	**48**	**0.00001-5**	**51**	**0.000005-0.2**	**10**	**NR**
Duration of Use						
Leave-On	*43*	*0.004-5*	*48*	*0.000005-0.1*	*10*	*NR*
Rinse-Off	*5*	*0.00001-0.006*	*3*	*NR*	*NR*	*NR*
Diluted for (Bath) Use	*NR*	*NR*	*NR*	*0.2*	*NR*	*NR*
Exposure Type						
Eye Area	7	0.00001-0.2	4	NR	NR	NR
Incidental Ingestion	NR	NR	NR	NR	NR	NR
Incidental Inhalation-Spray	NR	1[a]	1[a]	0.01[a]	NR	NR
Incidental Inhalation-Powder	NR	NR	NR	NR	NR	NR
Dermal Contact	48	0.00001-5	48	0.000005-0.2	10	NR
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	NR	NR	NR	NR	NR	NR
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	NR	NR	0.2	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR

Table 5. Frequency and concentration of use according to duration and type of exposure

	# of Uses[15]	*Max. Concs. of Use (%)*[16]	*# of Uses*[15]	*Max. Concs. of Use (%)*[16]	*# of Uses*[15]	*Max. Concs. of Use (%)*[16]
	Dextran Sulfate		**Sodium Dextran Sulfate**		**Sclerotium Gum**	
Totals	**9**	**0.01-0.1**	**9**	**0.005-0.5**	**193**	**0.003-2**
Duration of Use						
Leave-On	*9*	*0.01-0.1*	*9*	*0.005-0.5*	*155*	*0.003-2*
Rinse Off	*NR*	*NR*	*NR*	*NR*	*37*	*0.003-1*
Diluted for (Bath) Use	*NR*	*NR*	*NR*	*NR*	*1*	*0.003*
Exposure Type						
Eye Area	9	NR	2	0.01-0.2	25	0.2-0.8
Incidental Ingestion	NR	NR	NR	NR	NR	NR
Incidental Inhalation-Spray	NR	NR	NR	NR	13[a]	0.003
Incidental Inhalation-Powder	NR	NR	NR	NR	3	NR
Dermal Contact	9	0.01-0.1	9	0.005-0.5	168	0.003-2
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	NR	NR	NR	NR	20	0.003-1
Hair-Coloring	NR	NR	NR	NR	NR	0.8
Nail	NR	NR	NR	NR	1	0.6
Mucous Membrane	NR	NR	NR	NR	12	0.003-0.5
Baby Products	NR	NR	NR	NR	3	NR

	Hydrolyzed Sclerotium Gum		**Beta-Glucan**		**Sodium Carboxymethyl Beta-Glucan**	
Totals*	**NR**	**1**	**137**	**0.000001-0.1**	**67**	**0.0001-0.1**
Duration of Use						
Leave-On	*NR*	*1*	*103*	*0.0002-0.1*	*56*	*0.0002-0.1*
Rinse-Off	*NR*	*NR*	*34*	*0.000001-0.03*	*11*	*0.0001-0.1*
Diluted for (Bath) Use	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
Exposure Type						
Eye Area	NR	NR	9	NR	6	0.04
Incidental Ingestion	NR	NR	2	NR	NR	NR
Incidental Inhalation-Spray	NR	NR	NR	NR	NR	NR
Incidental Inhalation-Powder	NR	NR	7	NR	NR	0.02
Dermal Contact	NR	1	128	0.0002-0.1	66	0.0001-0.1
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	NR	NR	4	0.000001	NR	0.04
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	NR	8	0.03	NR	0.1
Baby Products	NR	NR	12	NR	NR	0.02

	Pullulan		**Rhizobian Gum**		**Hydrolyzed Rhizobian Gum**	
Totals*	**45[d]**	**0.03-17[e]**	**5**	**NR**	**4**	**0.4-3**
Duration of Use						
Leave-On	*38*	*0.2-12*	*4*	*NR*	*3*	*0.4-3*
Rinse-Off	*1*	*0.03*	*1*	*NR*	*1*	*NR*
Diluted for (Bath) Use	*NR*	*NR*	*NR*	*NR*	*NR*	*NR*
Exposure Type						
Eye Area	10	3	2	NR	2	3
Incidental Ingestion	6	17[e]	NR	NR	NR	NR
Incidental Inhalation-Spray	1[a]	NR	NR	NR	NR	NR
Incidental Inhalation-Powder	NR	NR	NR	NR	NR	NR
Dermal Contact	36	0.2-3	5	NR	4	0.4-3
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	NR	12	NR	NR	NR	NR
Hair-Coloring	NR	0.03	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	6	17[d]	NR	NR	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR

Table 5. Frequency and concentration of use according to duration and type of exposure

	# of Uses[15]	Max. Concs. of Use (%)[16]	# of Uses[15]	Max. Concs. of Use (%)[16]	# of Uses[15]	Max. Concs. of Use (%)[16]
	Alcaligenes Polysaccharides					
Totals*	**15**	0.005-0.3				
Duration of Use						
Leave-On	*13*	*0.3*				
Rinse-Off	*2*	*0.005*				
Diluted for (Bath) Use	*NR*	*NR*				
Exposure Type						
Eye Area	2	NR				
Incidental Ingestion	NR	NR				
Incidental Inhalation-Spray	NR	NR				
Incidental Inhalation-Powder	NR	NR				
Dermal Contact	15	0.005-0.3				
Deodorant (underarm)	NR	NR				
Hair - Non-Coloring	NR	NR				
Hair-Coloring	NR	NR				
Nail	NR	NR				
Mucous Membrane	NR	NR				
Baby Products	NR	NR				

***** Because each ingredient may be used in cosmetics with multiple exposure types, the sum of all exposure types my not equal the sum of total uses.
NR – no reported uses
[a] includes suntan products, in that it is not known whether or not the reported product is a spray
[b] this product is a pump spray
[c] it is not known whether or not the product is a spray
[d] the total of uses does not equal the duration of use for leave-on, rinse-off, and diluted for bath use because 6 uses are in oral hygiene products
[e] the use at 17% is in a breath freshener that dissolves in the mouth

Table 6. Ingredients Not Reported to be Used

Hydroxypropyl Xanthan Gum
Undecylenoyl Xanthan Gum
Xanthan Hydroxypropyltrimonium Chloride
Welan Gum
Biosaccharide Gum-3
Biosaccharide Gum-5
Pseudoalteromonas Exopolysaccharides
Carboxymethyl Dextran
Dextran Hydroxypropyltrimonium Chloride
Beta-Glucan Hydroxypropyltrimonium Chloride
Beta-Glucan Palmitate
Hydrolyzed Beta-Glucan
Oxidized Beta-Glucan
Myristoyl Pullulan
Levan

Table 7. Examples of Non-Cosmetic Uses

Ingredient	Non-Cosmetic Use
Xanthan Gum	direct food additive[24]; indirect food additive[26]; stabilizer, thickener, and emulsifying agent in water-based pharmaceutical preparations[33]; used in oil and gas drilling and completion fluids[35]; stabilizer in the agrochemical industry and in water based paints and water-based printing inks, and other industrial uses.[103]
Gellan Gum	direct food additive[25]; thickener and gelling agent in the food industry[104]; novel drug-delivery vehicle, film formation for transdermal drug delivery, component in controlled-release systems[13]; alternative to agar for microbiological media[11,104,105] and plant tissue culture[104]
Welan Gum	thermostable thickener for industrial and oilfield application[11]; suspending, stabilizing, emulsifying, and thickening agent for food, coating materials, medicine, concrete additives, and enhanced oil recovery[73]
Dextran	GRAS indirect food additive[28]; approved active ingredient for OTC use (as dextran 70) as an ophthalmic demulcent at 0.1% when used with another approved polymeric demulcent[34]; a plasma volume expander (as dextran 70) and as a blood flow adjuvant (as dextran 40)[35]; ^{99m}Tc-labeled dextran is used as a tracer in lymphoscintigraphy[49]
Sodium Dextran Sulfate	clinical reagent[35]
Sclerotium Gum	pharmaceutical applications include use in table coatings, ophthalmic solutions, and injectable antibiotic solutions; thickening in the oil industry; drilling's mud and enhanced oil recovery; preparation of adhesives, water colors, printing inks; preparation of liquid animal feed[8]
Beta-Glucan	direct food additive (as curdlan)[27]
Pullulan	glazing agent, as a film-forming agent, as a thickener, and as a carrier in the production of capsules for dietary supplements, coatings for coated tablets, and edible flavored films[31]; can be used in wound-healing compositions; denture adhesive; photographic, lithographic, and electronic applications[96]
Levan	agricultural applications[7]

Table 8. Acute toxicity studies

Ingredient	Animals	No./Group	Vehicle	Concentration/Dose/Protocol	LD_{50}/Results	Reference
ORAL						
Xanthan Gum	mice	not specified	water	not specified	>1 g/kg	106
Xanthan Gum	rats	not specified	not specified	5 g/kg, max	>5 g/kg	107
Xanthan Gum	dogs	not specified	not specified	20 g/kg, max	>20 g/kg	107
Gellan Gum	rats, M/F	not specified	not specified	administered in diet and by gavage; non-acetylated; >95% polysaccharide	>5 g/kg	39
Beta-Glucan (as curdlan)	mice, M/F	not specified	water	tested as a 10% suspension	>10 g/kg	42
Beta-Glucan (as curdlan)	rats, M	not specified	water	tested as a 10% suspension	>10 g/kg	42
Beta-Glucan (highly pure extract of *S. cerevisiae*)	rats	5M/5F	water	100 mg/ml suspension administered at 2 g/kg bw (20 ml/kg bw) by gavage	>2 g/kg	108
Sodium Carboxymethyl Beta-Glucan (>90% pure)	ddy mice	6M/6F	purified water	2 g/kg of a 20 aq. solution	>2 g/kg; no signs of toxicity	109
Pullulan	mice	not specified	olive oil	not specified	>14 g/kg	110
INHALATION						
Xanthan Gum	albino rabbits	5	none	calculated chamber conc of 21 mg/l; 1 h exposure	>21 mg/l; no toxicity; no gross lesions at necropsy	38
Gellan Gum	rats, M/F	not specified	not specified	non-acetylated; >95% polysaccharide; duration of exposure not stated	>5.09 mg/l	39
Beta-Glucan (as curdlan)	guinea pigs	5	not specified	100 µg/ml curdlan for 4 h; continuous flow aerosol	no inflammatory cell response	53
Beta-Glucan (as curdlan)	guinea pigs	3	NaOH	1 µg/ml; continuous flow for 4 h	decrease in inflammatory cells with curdlan alone, increase in cells, especially neutrophils with NaOH	54
Beta-Glucan (as curdlan)	guinea pigs	5	not specified	6 µg/ml for 40 min, and animals were examined 4 or 24 h after exposure, orr 1 µg/ml for 4 h and animals were examined 5 or 9 days after exposure	in lung lavage: decrease in lymphocytes at 24 h in lung wall: statistically significant decrease in macrophages after 5 days; decrease in lymphocytes for 1-7 days	54
Beta-Glucan (as curdlan)	guinea pigs	5	not specified	100 µg/ml; continuous flow for 40 min or 4 h	in in lung lavage: slight decrease in macrophages; (almost significant) decrease lymphocytes	54
Beta-Glucan (as curdlan); MMAD 5 µm	guinea pigs	not specified	dust	office dust spiked with 1% beta-glucan (as curdlan; 280 g office dust was spiked with 2.8 g curdlan) for 4 h in a whole-body exposure chamber; necropsied 5 or 18 h after exposure	glucan-spiked dust produced a delayed subacute nasal congestion in guinea pigs compared to dust without beta-glucan; at 18 h after exposure, there was a significant decrease in in nasal volume	52
Beta-Glucan (as curdlan)	**humans**	36	dust	addition of 10 mg beta-glucan (as curdlan) to office dust (10 mg curdlan/g dust); subjects were exposed to the dust in a climate chamber for 180 min	compared to "clean" dust, nasal volume decreased, swelling in the nasal turbinate increased, and nasal eosinophil cell concentration increased	55
Pullulan	guinea pigs	5	not specified	100 µg/ml curdlan for 4 h; continuous flow aerosol;	no inflammatory cell response	53
PARENTERAL						
Dextran Sulfate, avg MW of 7500, 47,000, and 458,000	mice and rats	not specified	not specified	0.1-2 g/kg, i.v. and i.p.	"some animals" died immediately; the largest MW dextran was more toxic	111
Beta-Glucan (as curdlan)	mice. M/F	not specified	saline	5% suspension, i.p.	males: 2.75 g/kg females: 2.5 g/kg	42
Beta-Glucan (as curdlan)	rats, M	not specified	saline	5% suspension, i.p.	2.75 g/kg; adhesions of beta-glucan to liver and spleen	42

Table 8. Acute toxicity studies

Ingredient	Animals	No./Group	Vehicle	Concentration/Dose/Protocol	LD_{50}/Results	Reference
Beta-Glucan (soluble; extract of *S. cerevisiae*)	ICR/HSD mice	not specified	not specified	≤1 g/kg, i.v.	>1 g/kg	[112]
Beta-Glucan (soluble; extract of *S. cerevisiae*)	Sprague-Dawley rats	not specified	not specified	0.5 g/kg, i.v.	>0.5 g/kg	[112]

Table 9. Repeated Dose Toxicity Studies

Ingredient	Animals/Group	Study Duration	Vehicle	Dose/Concentration	Results	Reference
			ORAL			
Xanthan Gum	rats (#/group not stated)	18 days	in diet	7.5%	paired feeding study – wt gains were similar to controls	106
Xanthan Gum	25 rats	30 days	in diet	1.5%	avg. wt of test animals was greater than controls; no gross lesions	58
Xanthan Gum	albino rats, 5M	91 days	in diet	3, 6, or 15%	reduced weight gain in the 15% group; no effect on organ wts; no microscopic lesions at necropsy in the 15% group	106
Xanthan Gum	rats, 5M/5F	110 days	in diet	0, 2.5, 5.0, or 10%	(the gum product consisted of drum-dried whole fermentation medium (beer)); no significant toxic effects	38
Xanthan Gum	CD rats, 30M/30F	2 yr	in diet	0, 0.25, 0.50, or 1.0 g/kg bw/day	no significant differences in growth rate, survival, hematology and clinical chemistry parameters, or organ weight were observed between treated and control animals; while not statistically significant (stat. sig.), there was an increase in uterine polyps in the high dose groups compared to controls, 5 in the high dose animals vs. 2 in controls	61
Xanthan Gum	beagle dogs, 3M/3F	12 wks	in diet	0, 0.25,or 0.5 g/kg bw/day	growth of high-dose males was slightly less than controls; the no-observable adverse effect level (NOAEL) was 0.25 g/kg bw/day	38
Xanthan Gum	beagle dogs, 2M/2F	12 wks	in diet	0, 1,or 2 g/kg bw/day; positive controls were given 20 g/kg bw/day powdered cellulose	immediate and persistent diarrhea in the 2 g/kg group; body wt loss was observed in treated and control animals, with the wt loss greatest in the 2 g/kg group; red blood cell counts, hemoglobin, and serum cholesterol levels were decreased in high dose animals; adrenal glands were slightly enlarged in the 2 g/kg group; no treatment-related microscopic lesion were observed at necropsy	113
Xanthan Gum	beagle dogs, 4M/4F	107 wks	in diet	0, 0.25, 0.37, and 1.0 g/kg/day	no significant differences in survival, body weight gain, feed consumption, organ weights, hematology parameters, or gross or microscopic lesions were observed between treated and control animals; a dose-related increased in urinary specific gravity was observed; ophthalmic findings were not considered treatment-related	61
Gellan Gum	20 rats	28 days	in diet	5%	no changes in urinalysis values; no gross lesions at necropsy	114
Gellan Gum; non-acetylated; >95% polysaccharide	Sprague-Dawley rats, 20M/20F	13 wks	in diet	0-6%	no mortality; no signs of toxicity	39
Gellan Gum; varied degree of acetylation; 58.5% polysac.	beagle dogs, 5M/5F	52 wks	in diet	0, 3, 4.5, or 6%	no mortality; feed consumption was greater in treated animals compared to controls; no adverse effects were observed	39
Gellan Gum; varied degree of acetylation; 58.5% polysac.	rhesus monkey, 2M/2F	28 days	not provided	0, 1, 2, or 3 g/kg, by gavage	no signs of toxicity	39
Dextran	albino rats, 6M	62 days	in diet	15%	wt gain was normal	106
Beta-Glucan (as curdlan)	CD-1 mice, 10M/10F	8 wk	in diet	0, 1, 5, 10, 20, or 30%; equiv to 0, 1.4, 7.1, 14, 29, and 43 g/kg bw, respectively	one female of the 30% group died; body wt gains of male mice of the 30% group were decreased compared to controls; no gross abnormalities; differences in stools and cecal wts were reported; the NOEL was 5% based on an increase in full cecal wts and large stools at higher doses	42
Beta-Glucan (as curdlan)	CD-1 mice, 100M/100F	99-114 wks (until survival was 20%)	in diet	0, 1, 5, or 15%	no treatment-related differences in survival or body wts; feed consumption was decreased by ~13% in 15% females; NOEL of 5% due to decreased feed consumption	42

Table 9. Repeated Dose Toxicity Studies

Ingredient	Animals/Group	Study Duration	Vehicle	Dose/Concentration	Results	Reference
Beta-Glucan (as curdlan)	Sprague-Dawley Ta rats; 5M	4 wks	in diet	0, 3, 10, or 30%; equiv to 0, 2.5, 8.5,or 30 g/kg bw 30% powder agar group	no difference in body wts; dose-related increase in neutrophils, with a stat. sig. increase in the 10% group; occult blood was present in the urine 4 10% rats, 1 30% rat, and 1 fed agar; relative (rel.) kidney wts were statistically significantly decreased in all groups; rel. liver weights were statistically significantly decreased and pituitary wts were stat. sig. increased in the 30% group	42
Beta-Glucan (as curdlan)	20 Sprague-Dawley rats, males	8 wks	in diet	0, 1, 5, or 15%	no mortality or signs of toxicity; body wt of animals of the 15% dose group were stat. sig. decreased; feed consumption in this group was slightly decreased	42
Beta-Glucan (as curdlan)	Sprague-Dawley rats, 10M/10F	3 mos	in diet	0, 5, 10, or 20%; equiv to 0, 4.4, 9, and 19 g/kg bw (males) and 0, 5.5, 12, and 24 g/kg bw (females)	body wt gains decreased with increasing dose, being stat. sig. in the 20% group; a stat. sig. decrease in platelet count in males and in total protein and globulin concs. in males and females of the 10 and 20% groups; stat. sig. increase in body wt of males of the 10 and 20% group; stat. sig. decreases in absolute liver wts in 20% males, absolute (abs.) kidney wts of 10 and 20% males, abs. and rel. ovary wts in 20% females, abs. and rel. pituitary wts of in all females of all doses; and rel. adrenal wts in 10 and 20% males; differences in stools and cecal wts were reported; the NOEL was 5% based on fecal changes, diarrhea, and cecal wts and large stools at higher doses	42
Beta-Glucan (as mushroom beta-glucan from *Ganoderma lucidum*)	CD (SD) IGS rats, 12M,12F	3 mos	sterile water; by gavage	0, 500, 1000, or 2000 mg/kg bw (10 ml/kg dosing volume)	no test article-related adverse effects on mortality, toxicity; opthalmoscopy, body wts or body wt gains, clinical chemistry, hematology, or urinalysis; statistically significant decreases in testes wt in males of the low dose group and heart wts in females of the high dose group were not considered test-article related; there were no treatment related gross or microscopic lesions, the NOAEL was 2000 mg/kg bw	42
Beta-Glucan (as curdlan)	CD rats; 60M/60F	2 yrs	in diet	0, 1, 5, or 15%	no changes in mortality, behavior, appearance, or ophthalmic parameters; wt gain was decreased by ~10%, which was not stat. sig., in the 15% group; feed consumption was also reduced; no microscopic lesions were found the NOEL was 5% based on decreased feed consumption, body wt gain and increased cecal wts	42
Beta-Glucan (as curdlan)	CD rats; 60M/60F, of the F_{1a} generation of a reproductive tox. study	124-127 wks (20% survival)	in diet	0, 1, 5, or 15%	no changes in mortality, behavior, appearance, or ophthalmic parameters; body wts of the 15% group were stat. sig. decreased, and body wts were stat. sig. decreased in 5% males until wk 65; feed consumption was decreased in the 15% group; no changes in hematology or urinary parameters, but some stat. sig. changes were reported in blood chemistry; stat. sig increase in gross and microscopic incidences of uterine polyps in the 15% groups – the incidences were 0/450, 3/50, 4/51, and 7/50 for the 0, 1, 5, and 15% groups, indicating that the polyps were possible treatment-related; the NOEL was 1% based on increased cecal wts, decreased body wts and feed consumption, and the incidence of polyps	42
Beta-Glucan (as curdlan)	beagle dogs, 4M/4F	52 wks	in diet	0, 1, 5, or 15%	one 15% male died at 37 wks (not dose related); no stat. sig. treatment-related changes, except for fecal changes and cecal wts; NOEL was 5%based on fecal changes and cecal wts	42

Table 9. Repeated Dose Toxicity Studies

Ingredient	Animals/Group	Study Duration	Vehicle	Dose/Concentration	Results	Reference
Beta-Glucan (highly pure extract of *S. cerevisiae*)	SPF Fischer rats, 10M/10F	91 days	water	0, 2, 33.3, or 100 mg/kg bw/day, volume 0.5 ml/100 g bw, by gavage	no mortality; no stat. sig. differences in wt gains, feed consumption, or gross or microscopic lesions; a dose-dependent and stat. sig. increase in clotting time in males, isolated stat. sig. changes in some clinical chemistry parameters, and slight but stat. sig. incr. in absolute liver, kidney, heart, spleen, adrenal, and testicle wts in males and absolute kidney and thymus wts in females were not considered toxicologically significant; NOAEL was 100 mg/kg bw/day	108
Beta-Glucan (high-purity extract barley beta-glucan)	SPF Wistar rats, 5M/5F	28 days	in diet	0, 1, 5, or 10%, supplemented with ≤10% potato starch	no mortality; no sig. effects on body wts, feed consumption, or functional observational battery results; no treatment-related change in hematology or urinalysis values; empty caecum wt was increased	115
Beta-Glucan (extracted from *Candida albicans*)	Sprague-Dawley rats, 20M/20F	52 wks	sterile saline (using a rubber catheter)	0, 50, 100, or 200 mg/kg/day	no sig. effects on mortality, body wts, feed consumption, or hematology, clinical chemistry, or urinalysis parameters; with the exception of cecal enlargement with variable hyperplasia, not gross or microscopic lesions were noted; the NOEL was 100 mg/kg/day	116
Pullulan	8 Wistar rats, males (test and cellulose control groups)	4 or 9 wks	in diet	0, 5, 10, 20, or 40%; equiv. to 0, 2500, 5000, 10,000, or 20,000 mg/kg, respectively cellulose controls: 20 or 40% cellulose	body wt gains were decreased by day 10 in rats of the 20 and 40% groups when compared to untreated controls; wt gains of animals of the 5 and 10% pullulan group were lower than untreated controls after 7 wks, but this difference was not stat. sig.; similar decreases were observed in the animals fed cellulose; diarrhea was observed with 40% pullulan; rel. wts of the stomach, small intestine, and large intestine were increased in treated animals	43,44
Pullulan (200,000 MW)	SPF Wistar rats, 10M/10F	13 wks	in diet	0, 2.5, 5, or 10%,; equiv to 0, 1960, 4100, or 7900 mg/kg bw/day diet of the groups fed 0, 2.5, or 5% pullulan was supplemented with 10, 7.5, or 5% potato starch, respectively	no mortality; no dose-related clinical sign; stat. sig. reduced motor activity in females of the 5 and 10% groups was observed and appeared treatment related, but as a physiological phenomenon rather than a toxic effect; no difference in hematology parameters between treated and control groups; differences that were observed in clinical chem. parameters were not biologically significant or dose-related; dose-dependent, stat. sig. increases in abs. and rel. empty cecal wts were observed in males of the 5 and males and females of the 10% group; no microscopic changes were observed	44
Pullulan	Sprague-Dawley rats, 15M/15F	62 wks(was to be 24 mos; study was terminated because of poor survival due to pneumonia)	in diet	0, 1, 5, and 10%	no treatment-related effects on body wts, feed consumption or organ wts (except for cecal wts); changes in hematology or clinical chemistry parameters and microscopic lesions that were observed were not considered treatment-related; the NOAEL was 10% in the diet (equiv. to 4450 mg/kg bw/day)	117
				INHALATION		
Beta-Glucan (as curdlan)	guinea pigs, 16F	5 wks	distilled water	100 µg/ml; 4 h/day, 5 days/wk	no significant change in lung lavage cells, but there was a decrease in the number of lymphocytes; slight but not statistically significant increase in macrophages and eosinophils in the lung wall cells; no lesions observed at microscopic examination of the lungs	118
Beta-Glucan (as curdlan)	guinea pigs, 6	5 wks	saline	100 µg/ml; 4 h/day, 5 days/wk; continuous flow exposure with a dose of 8 pg; animals were examined 24 h after last dose	the only effect on lung lavage cells was a slight decrease in neutrophils; there was no effect on the number of lung wall cells	119
				PARENTERAL		
Xanthan Gum	mice	2 wks	water	5 mg in 0.5 ml water; 10 i.p. injections over 2 wks	no toxicity; there was no xanthan gum in the abdominal cavity at necropsy	106

Table 9. Repeated Dose Toxicity Studies

Ingredient	Animals/Group	Study Duration	Vehicle	Dose/Concentration	Results	Reference
Dextran, partly hydrolysed, bacterial, 75,000 avg MW	16	103-113 wks	physiological saline	30 ml of 6% solution, i.v.	4 animals died and one was killed in moribund condtion; wt gain was similar to that of controls; increases in absolute heart and adrenal wts were probably statistically significant; statistically significant increase in liver and spleen wts; no microscopic lesions	47
Beta-Glucan (soluble; extract of *S. cerevisiae*)	mice	7 days	not specified	250 mg/kg bw, i.p.	no effect on weight gain	112
Beta-Glucan (soluble; extract of *S. cerevisiae*)	guinea pigs	7 days	not specified	250 mg/kg bw, i.p.	sig. 10% decrease in weight gain	112
Beta-Glucan (soluble; extract of *S. cerevisiae*)	ICH/HSD mice, M	30 days	not specified	≤1000 mg/kg bw, i.v.	sig. toxicity leading to hepatosplenomegaly in the 40 and 1000 mg/kg bw groups	112
Beta-Glucan (soluble; extract of *S. cerevisiae*)	ICH/HSD mice, M	60 days	not specified	≤1000 mg/kg bw, i.v.	dose-dependent increase in hepatosplenomegaly; was stat. sig. at 1000 mg/kg	112

Table 10. Genotoxicity studies

Ingredient	Concentration	Vehicle	Procedure	Test System	Results	Reference
			IN VITRO			
Gellan Gum (non-acetylated; >95% polysacc.)	10, 30, 100, 300, and 1000 µg/plate	not provided	Ames test, with and without metabolic activation	*Salmonella typhimurium* TA98, TA100, TA1537, TA1538, TA1535	negative	39
Gellan Gum (non-acetylated; >95% polysacc.)	3, 5, 10, and 20 mg/ml	not provided	DNA repair test (details not provided)	rat hepatocytes	negative	39
Gellan Gum (non-acetylated; >95% polysacc.)	3, 5, 10, and 20 mg/ml	not provided	V79/HGRPT (details not provided)	Chinese hamster lung fibroblasts	negative	39
Sodium Dextran Sulfate (54,000 MW)	1.0, 7.5, 25 mg/plate	not provided	Ames test; appropriate positive controls were used	S. typhimurium TA100, TA98	negative	120
Sodium Dextran Sulfate (54,000 MW)	0, 10, 100 µg	distilled water	hepatocyte primary culture (HPC)/DNA repair test; appropriate positive controls were used	rat hepatocytes	negative	120
Sodium Dextran Sulfate (54,000 MW)	0, 10, 100 µg	distilled water	intestinal mucosal cell (IMC)/DNA repair test; appropriate positive controls were used	intestinal mucosal cells from rat ileum or colon and rectum	negative	120
Beta-Glucan (as curdlan)	15-5000 µg/plate	sterile water	Ames test, with and without metabolic activation	*S. typhimurium* TA98, TA100, TA1537, TA1538, TA1535	negative	42
Beta-Glucan (as mushroom beta-glucan from *Ganoderma lucidum*)	313-5000µg/plate	not provided	Ames test, with and without metabolic activation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537		121
Beta-Glucan (as curdlan)	625-5000 µg/ml	tissue culture medium	chromosomal aberration assay, with and without metabolic activation	Chinese hamster ovary (CHO) cells	negative	42
Beta-Glucan (as mushroom beta-glucan from *Ganoderma lucidum*)	313-5000µg/ml	culture medium	chromosomal aberration assay, with and without metabolic activation	CHO cells	negative	121

Table 10. Genotoxicity studies

Ingredient	Concentration	Vehicle	Procedure	Test System	Results	Reference
Beta-Glucan (as curdlan)	12.5-5000 µg/ml	tissue culture medium	tk locus test, with and without metabolic activation	mouse lymphoma L518Y cells	negative	[42]
Sodium Carboxymethyl Beta-Glucan	0, 3-5000 µg/plate	Milli-Q water	Ames test, with and without metabolic activation, appropriate positive controls were used;	*S. typhimurium* TA98, TA100, TA1537, TA1535	negative	[122]
Sodium Carboxymethyl Beta-Glucan	0, 100-5000 µg/plate	Milli-Q water	Ames test, with and without metabolic activation; appropriate positive controls were used	*S. typhimurium* TA98, TA100, TA1537, TA1535	negative	[122]
Sodium Carboxymethyl Beta-Glucan	0, 195-50,000 µg/ml	purified water	Ames test, with and without metabolic activation; appropriate positive controls were used;	*S. typhimurium* TA98, TA100, TA1537, TA1535; *Escherichia coli* WP2 uvrA	negative	[123]
Sodium Carboxymethyl Beta-Glucan	0, 3125-50,000 µg/ml	purified water	Ames test, with and without metabolic activation; appropriate positive controls were used	*S. typhimurium* TA98, TA100, TA1537, TA1535; *Escherichia coli* WP2 uvrA	negative	[123]
Carboxymethyl-Glucan	12.5, 25, 50, 100, and 200 µg/ml		electrophoresis test in single-cell gel (comet assay)	CHO-k1 cells	negative	[124]
Pullulan	≤10,000 µg/plate	not provided	Ames test, with and without metabolic activation; appropriate positive controls were used	*S. typhimurium* TA98, TA100, TA1537, TA1535; *Escherichia coli* WP2 uvrA	negative	[117]
Pullulan	12 mg/ml	not provided	chromosomal aberration assay	Chinese hamster lung fibroblasts	negative	[125]
Pullulan	20 mg/plate	distilled water	rec assay; with and without metabolic activation	Bacillus subtilis	weak positive	[126]
			IN VIVO			
Beta-Glucan (as curdlan)	0, 500, 1000, or 2000 mg/kg	water	micronucleus test; dosed by gavage at 24 h intervals; number of doses not specified; followed OECD Guideline No. 474	male and female CD-1 mice	negative	[42]
Beta-Glucan (as mush-room beta-glucan from *Ganoderma lucidum*)	0, 1250, 2500, and 5000 mg/kg bw	sterile water	micronucleus test; single dose by gavage ; blood samples were taken 24, 48, and 72 h after dosing	CD-1 mice, 5/sex/group	negative	[121]
Pullulan	1800 mg/kg bw.	not provided	micronucleus test; one i.p. injection	ddy mice	negative	[127]
Pullulan	4x1000 mg/kg bw	not provided	micronucleus test; four i.p. injections in 24 h	ddy mice	negative	[127]

Table 11. Dermal Irritation and Sensitization

Ingredient	Animals/Subjects/Gp	Dose/Conc/Vehicle	Procedure	Results	Reference
			IRRITATION - NON-HUMAN		
Xanthan Gum	rats	1%; vehicle not provided	solution was applied daily for 15 days (details not provided)	not an irritant	[38]
Xanthan Gum	6 rabbits	0.5 ml of a 1% solution	primary cutaneous irritation test; occlusive patches on intact and abraded skin	non-irritant; primary irritation index (PII) – 0.13	[128]
Xanthan Gum	6 rabbits	0.5 ml of a 1% solution	primary cutaneous irritation test; occlusive patches on intact and abraded skin	non-irritant; PII – 0.13	[128]
Xanthan Gum	3 rabbits	2 ml/animal of a 1% solution	6-wk cumulative cutaneous irritation test, 5 applications/wk	very well tolerated; mean max. irritation index - 0	[128]
Xanthan Gum	3 rabbits	1%	6-wk cumulative cutaneous irritation test, 5 applications/wk	very well tolerated; MMII - 0	[128]
Xanthan Gum	rabbits	5% aq.	daily applications to shaved skin (details not provided)	localized irritation with bleeding and cracking; the effects may have been due to continuous moisten-ing of the skin	[106]
Beta-Glucan	rabbits	not provided	primary skin irritation test (details not provided)	not an irritant	[91]
Beta-Glucan	rabbits	not provided	repeated skin irritancy test (details not provided)	not an irritant	[91]

Table 11. Dermal Irritation and Sensitization

Ingredient	Animals/Subjects/Gp	Dose/Conc/Vehicle	Procedure	Results	Reference
Sodium Carboxy-methyl Beta-Glucan (>90% pure)	Japanese white rabbits, 6M	0.5 g moistened with 0.2 ml distilled water	occlusive patches were applied to intact and abraded shaved skin for 24 h	non- to mildly irritating; primary irritation index (PII) of 0.33/8 at test site and 0.29/8 for adhesive control; no irritation was reported for intact skin	[129]
Sodium Carboxy-methyl Beta-Glucan (>90% pure)	3 guinea pigs	2, 10, or 50% (w/v) in distilled water	24-h occlusive patch test; this test was used to determine test concentration for the maximization study described below	slight skin irritation was observed at a concentration of 50%	[130]
			IRRITATION - HUMAN		
Beta-Glucan	not provided	not provided	occlusive patch test	no an irritant	[91]
Sodium Carboxy-methyl Beta-Glucan (>90% pure)	40 subjects; 27M/13F	applied neat; small amount of vaseline was used for adhesion	24-h occlusive patch test; 0.1 g of test material was applied	not a primary skin irritant; no irritation was observed	[131]
			SENSITIZATION – NON-HUMAN		
Xanthan Gum	guinea pigs, 18M	0.1%; vehicle not provided	intradermal challenge test; test solution was injected intracutaneously 3x/wk for 10 injections; the challenge was performed after a 10-day non-treatment period	not a sensitizer	[38]
Beta-Glucan	guinea pigs	not provided	skin sensitization test (details not provided)	not a sensitizer	[91]
Sodium Carboxy-methyl Beta-Glucan (>90% pure)	Hartley guinea pigs, 6F	10% in distilled water	maximization study using Freund's complete adjuvant and SLS; 0.1% aq. 2,4-dinitro-1-chlorobenzenze was used as the positive control dose volumes were 0.1 ml for intradermal induction, 0.2 ml for dermal induction, and 0.1 ml for challenge	not a sensitizer	[130]
			SENSITIZATION - HUMAN		
Beta-Glucan (as Curdlan)	213 subjects; M	dose not provided; was an aq. paste	modified Draize 'multiple insult' patch test; occlusive patches were applied every other day for 10 applications; a 48-h challenge was performed after a 10-14 day non-treatment period	trace, insignificant, irritation observed during induction; not a sensitizer	[42]
Sodium Carboxy-methyl Beta-Glucan	32 subjects; 8M/24F	induction and challenge: 2% in distilled water	induction: 9 24-h occlusive patches were applied challenge: occlusive patch applied after a 10-14 day non-treatment period to a previously unpatched site reactions were graded on a scale of 0-4	not a sensitizer during induction, 2 subjects had doubtful reactions and one had a grade 1 reaction	[132]
			PHOTOALLERGY- HUMAN		
Sodium Carboxy-methyl Beta-Glucan	8 male/24 female subjects	induction and challenge: 2% in distilled water	induction: 6 24-h patches were applied to non-tanned skin; the test site was irradiated with 2x MED UVB within 10 min after patch removal challenge: applied after a 10-14 day non-treatment period, a 24-h patch was applied to a previously unpatched site; the site was irradiated with 18 J/cm^2 UVA within 10 min after patch removal MULTITESTER light source was used; 0-4 scale used for scoring	not photoallergenic 31 subjects had grade 1 skin reactions, and on had a grade 2 reaction, to UVB exposure during induction 1 subject had a doubtful reaction after challenge	[132]

Table 12. Ocular Irritation

Ingredient	Animals/Subjects/Group	Concentration/Vehicle	Procedure	Results	Reference
			ALTERNATIVE STUDIES		
Sodium Carboxy-methyl Beta-Glucan		5% stock solution containing 2% Sodium Carboxymethyl Beta-Glucan	Ocular tolerance test using HET-CAM method	weakly irritant	[133]
			NON-HUMAN		
Xanthan Gum	rabbits	1%	ocular irritation test	non-irritating; acute ocular irritation index (AOII) - 2.50/110	[128]
Xanthan Gum	rabbits	1%	ocular irritation test	non-irritating; AOII – 5.83/110	[128]
Xanthan Gum	rabbits	1%	instilled in the conjunctival sac of rabbit eyes for 5 days (details were not provided)	not irritating	[38]
Gellan Gum	New Zealand White rabbits, 3M	0.2 or 0.3%	Draize study; 50 µl of the test solution was instilled into the conjunctival sac of the eye three times a day for 10 days	not irritating	[134]
Gellan Gum	albino rabbits	0.8%	details not provided	not irritating	[135]
Sodium Carboxy-methyl Beta-Glucan (>90% pure)	Japanese white rabbits, 3M	undiluted	0.1 mg were instilled into the conjunctival sac of one eye, and the contralateral eye served as an untreated control; the eyes of one group were rinsed 1 min after instillation	practically non-irritating; slight redness of the conjunctiva in both washed and unwashed eyes at 1 h, considered due to the powder	[136]
			HUMAN		
Gellan Gum	3 subjects	0.1-0.5%; vehicle not specified	25 µl of a gel formulation was instilled in the conjunctival sac of the eye, remained in contact with the eye for 9-52 min	not irritating	[134]

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Re-evaluation of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives

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Abstract

The present opinion deals with the re-evaluation of the safety of food-grade carrageenan (E 407) and processes Eucheuma seaweed (E 407a) used as food additives. Because of the structural similarities, the Panel concluded that processed Eucheuma seaweed can be included in the evaluation of food-grade carrageenan. Poligeenan (average molecular weight 10–20 kDa) has not been authorised as a food additive and is not used in any food applications. In its evaluation of carrageenan (E 407) and processed Eucheuma seaweed (E 407a), the Panel noted that the ADME database was sufficient to conclude that carrageenan was not absorbed intact; in a subchronic toxicity study performed with carrageenan almost complying with the EU specification for E 407 in rats, the no-observed-adverse-effect level (NOAEL) was 3,400–3,900 mg/kg body weight (bw) per day, the highest dose tested; no adverse effects have been detected in chronic toxicity studies with carrageenan in rats up to 7,500 mg/kg bw per day, the highest dose tested; there was no concern with respect to the carcinogenicity of carrageenan; carrageenan and processed Eucheuma seaweed did not raise a concern with respect to genotoxicity; the NOAEL of sodium and calcium carrageenan for prenatal developmental dietary toxicity studies were the highest dose tested; the safety of processed Eucheuma seaweed was sufficiently covered by the toxicological evaluation of carrageenan; data were adequate for a refined exposure assessment for 41 out of 79 food categories. However, the Panel noted uncertainties as regards the chemistry, the exposure assessment and biological and toxicological data. Overall, taking into account the lack of adequate data to address these uncertainties, the Panel concluded that the existing group acceptable daily intake (ADI) for carrageenan (E 407) and processed Eucheuma seaweed (E 407a) of 75 mg/kg bw per day should be considered temporary, while the database should be improved within 5 years after publication of this opinion.

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Keywords: Carrageenan, E 407, processed Eucheuma seaweed, E 407a, degraded carrageenan, food additives

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Summary

The present opinion deals with the re-evaluation of the safety of food-grade carrageenan (E 407) and processed Eucheuma seaweed (E 407a) used as food additives.

Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised as food additives in the European Union (EU) in accordance with Annex II and Annex III to Regulation (EC) No 1333/2008 on food additives and specific purity criteria have been defined in the Commission Regulation (EU) No 231/2012[1]. In the EU, these two food additives have been evaluated by the Scientific Committee for Food (SCF) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in different occasions. The SCF (1978), endorsed the acceptable daily intake (ADI) of 0–75 mg/kg body weight (bw) per day for carrageenan (E 407) previously established by JECFA (1974). JECFA in 1984 changed to an ADI 'not specified' (JECFA, 1984), and in 2002, a group ADI 'not specified' was allocated to the sum of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) (JECFA, 2002). The SCF maintained the original group ADI for all types of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) of 0–75 mg/kg bw because of some uncertainty 'about the general immuno-reactive potential of the various carrageenans now in use as food additives' (SCF, 1996). In 2003, the SCF had no objection to the use of carrageenan in follow-on formulae up to a maximum level of 0.3 g/L (SCF, 2003b). In its latest evaluation, JECFA in 2015, concluded that 'the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1,000 mg/L is not of concern' (JECFA, 2015).

According to the Commission Regulation (EU) No 231/2012, 'carrageenan (E 407) consists chiefly of the potassium, sodium, magnesium and calcium sulphate esters of galactose and 3,6-anhydrogalactose polysaccharides. The prevalent polysaccharides in carrageenan are designated as κ-, ι-, λ- depending on the number of sulphate by repeating unit (i.e. 1,2,3 sulphate)'. In the processed Eucheuma seaweed (E 407a), the main polysaccharide is κ-carrageenan.

Because of the structural similarities of processed Eucheuma seaweed and the conventionally processed food-grade carrageenan and the similarities of effects they caused in the comparative study (Documentation provided to EFSA n. 38), the Panel concluded that the re-evaluation of processed Eucheuma seaweed (E 407a) can be included in that of food-grade carrageenan (E 407).

According to one of the interested parties (Documentation provided to EFSA n. 19), carrageenan has a molecular weight distribution from 30 kDa to as high as 5,000 kDa and is defined as having a weight-average molecular weight between 200 and 800 kDa. Furthermore, it has been confirmed by industry (Documentation provided to EFSA n. 19) that commercial carrageenan (E 407) may have a weight-average molecular weight as low as 200 kDa. In view of the Panel, the molecular weight distribution of such a carrageenan product may have a considerable fraction of molecules encompassing weight-average molecular weight of degraded carrageenan.

The Panel noted that in the literature the information on the molecular weight in the individual carrageenan preparation is often unprecise mixing the terms 'molecular weight', 'weight-average molecular weight' and 'number-average molecular weight'.

The debate in literature with respect to the safety of native (undegraded) carrageenan has been related to the presence of degraded carrageenan. Degraded carrageenan is also known in forms of artificially produced products from carrageenan (e.g. poligeenan, C16) associated with adverse effects. Poligeenan is a much lower weight-average molecular weight polymer (10–20 kDa) generated by subjecting ι-carrageenan to the extreme conditions of acid hydrolysis at low pH (0.9–1.3) and high temperatures (> 80°C) for several hours (Cohen and Ito, 2006; McKim, 2014; JECFA, 2015; USDA, 2016). C16 is artificially formed from ι-carrageenan under conditions of acidic hydrolysis with 0.1 M sulfuric acid, at 60°C for 1.5 h (Glaxo Lab).

The Panel emphasised that degraded carrageenan (e.g. poligeenan or C16) has not been authorised as a food additive in the EU. Information available from the US indicated that poligeenan is not used in any food applications (USDA, 2016). The Panel further noted that the term 'poligeenan' has been used in literature including test protocols in a broader sense. However, more specifically poligeenan is a breakdown product of ι-carrageenan consisting of the same sulfated galactose and sulfated anydrogalactose units, linked by α-1,3- and β-1,4-glycosidic bonds as in all types of carrageenan. According to information provided from interested parties (Documentation provided to EFSA n. 18), poligeenan comprises polysaccharide molecules of molecular weight ranging from approximately 2 to 200 kDa, and the weight-average molecular weight of poligeenan ranges from 10 to 20 kDa. The Panel noted that based on the data provided (Documentation provided to EFSA n.

[1] Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) no 1333/2008 of the European Parliament and of the Council. OJ L 83, 22.3.2012, p. 1–295.

18) the overlapping between the low molecular weight tail of carrageenan and high molecular weight tail of poligeenan is increasing with the decrease of the weight-average molecular weight of the carrageenan preparation.

Uno et al. (2001a), in a survey of 29 samples of food-grade carrageenan representing κ-, ι- and λ-carrageenan determined a number-average molecular weight of 193–324 kDa and a weight-average molecular weight of 453–652 kDa. According to Uno et al. (2001a), 'no obvious peak of poligeenan was detected' and the 'detection limit' for poligeenan was about 5% in the sample of carrageenan.

According to information from one of the interested parties (Documentation provided to EFSA n. 19), 'in native carrageenan, this low molecular weight tail (LMT) region represents less than 10% of the total carrageenan molecule. Although there is a Commission specification of not more than 5% at less than 50 kDa, in practice there is no validated analytical method that can accurately quantify the percent of LMT present'. The Panel noted that, according to the interested parties, an interlaboratory validated analytical method to accurately measure the low molecular weight distributions of carrageenan is not fully developed or available at present.

The Panel noted that in most toxicity studies no adequate information on the molecular weight distribution of carrageenan was available. Therefore, it was not possible to assess whether the carrageenan tested complied with the EU specifications (specially the limit of low molecular weight carrageenan $<$ 5% below 50 kDa). Furthermore, the Panel noted the methodological limitation of *in vivo* studies, in which carrageenan was given in drinking water, which is not representative of the dietary exposure where food-grade carrageenan will be bound to protein. Therefore, the extent to which drinking water studies might be representative for food-grade carrageenan used as a food additive and consequently of the relevance of these studies for its safety assessment was deemed limited.

The available database on processed Eucheuma seaweed was limited to the endpoints of subchronic toxicity and genotoxicity but was sufficient for the evaluation of the similarities between processed Eucheuma seaweed and food-grade carrageenan.

Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised in a wide range of foods. The Panel did identify brand loyalty for specific food categories, e.g. flavoured fermented milk products for infants and toddlers, thus the Panel considered this scenario as the most appropriate and realistic scenario for risk characterisation for all population groups.

A maximum estimated exposure assessment scenario taking into account the food for special medical purposes for infants and young children (FC 13.1.5.1. Dietary foods for infants for special medical purposes and special formulae for infants, and FC 13.1.5.2. Dietary foods for babies and young children for special medical purposes as defined by Commission Directive 1999/22/EC) was also performed using food industry data to estimate exposure for infants and toddlers who may be on a specific diet. This exposure scenario considered products belonging to food categories 13.1.2 and 13.1.4 excluding food category 13.1.1 (infant formulae) and 13.1.3 (processed cereal-based foods) where carrageenan (E 407) and processed Eucheuma seaweed (E 407a), according to EU Regulation, are not authorised. Considering that this diet is required due to specific needs, it is assumed that consumers are loyal to the food brand, therefore, the maximum reported use level for the foods for special medical purposes (FSMP) was used and the mean of the typical reported for the remaining food categories.

The refined estimates were based on 41 out of 79 food categories in which carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised. Overall, the Panel considered that the uncertainties identified would, in general, result in an overestimation of the real exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives in European countries for both the maximum level exposure scenario and for the refined exposure assessment scenarios when considering only food additives uses for which data have been provided.

No information on the usage levels of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) has been reported to EFSA for 19 food categories. However, the Panel noted that, given the information from the Mintel Global New Products Database (GNPD), it may be assumed that carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are used in several food categories (n = 7) for which no data have been provided by food industry. The Panel noted that out of these 19 food categories, pasta and breakfast cereals are products highly consumed. If this would be confirmed, it would therefore result in an underestimation of the exposure.

For FSMP, the Panel noted that one industry reported a use of carrageenan as food additive at the level of the maximum permissible level (MPL) (300 mg/kg), according to the Annex II to Regulation (EC) No 1333/2008, in one niche product which is not align with information reported from the Mintel

GNPD (Appendix D), where no FSMP products seem to be labelled on the market. If this information is confirmed, the uncertainties arising from the FSMP exposure scenario would lead to an overestimation of the current exposure to carrageenan products as food additives calculated by the Panel.

Concerning uses of carrageenan in food for infants and young children the Panel concurred with the SCF (1998a, 2003a,b) 'In the absence of any further information on possible absorption of carrageenan by the immature gut in the very young infant, the Committee reaffirms its earlier view (SCF, 1998a) that it remains inadvisable to use carrageenan in infant formulae that are fed from birth, including those in the category of foods for special medical purposes. The Committee has no objection to the use of carrageenan in foods for older infants, such as follow-on milks (SCF, 1983) and weaning foods (SCF, 2003a)'.

In its evaluation of carrageenan (E 407) and processed Eucheuma seaweed (E 407a), the Panel noted that:

- according to industry carrageenan (E 407) is defined as having a weight-average molecular weight of 200–800 kDa;
- Uno et al. (2001a) determined in a survey on samples of food-grade carrageenan, representing κ-, ι- and λ-carrageenan, a weight-average molecular weight of 453–652 kDa and detected no obvious peak of poligeenan with a detection limit for poligeenan of about 5%;
- the ADME database was sufficient to conclude that high molecular weight carrageenan (e.g. κ/λ-carrageenan with a number-average molecular weight of approx. 800 kDa, study in rhesus monkeys) was not absorbed intact, while low molecular weight carrageenan (number-average molecular weight: 88 kDa or less) was found in tissues of rats or guinea pigs after administration of this material by gavage or diet;
- in one subchronic toxicity good laboratory practice (GLP) study in rats performed with κ-carrageenan with an average molecular weight range of 196–257 kDa (not specified if number- or weight-average molecular weight; LMT of 1.9–12% < 50 kDa (mean 7%)), almost complying with the EU specification, the no-observed-adverse-effect level (NOAEL) was equal to 3,394–3,867 mg/kg bw per day for males and females, respectively, the highest dose tested;
- no adverse effects have been detected in several chronic toxicity studies in rats with carrageenan (mostly κ/λ-type, no adequate indication of molecular weight distribution); from the available rat studies, NOAELs up to 7,500 mg/kg bw per day, the highest dose tested, were identified (Nilson and Wagner, 1959). However, in another study in rats given carrageenan preparations with a molecular weight of 244 and 252 kDa, a lowest-observed-adverse-effect level (LOAEL) of 1% in the diet (equivalent to 500 mg/kg bw per day) was identified by the Panel (Documentation provided to EFSA n. 43). The Panel noted that the characterisation of the test material in all the chronic studies was limited;
- carrageenan (different types) and processed Eucheuma seaweed did not raise a concern with respect to genotoxicity;
- there was no concern with respect to carcinogenicity for carrageenan (mostly κ/λ-type);
- the NOAEL of sodium and calcium κ/λ-carrageenan for developmental effects found in dietary prenatal developmental toxicity studies was 3,000 and 5,000 mg/kg bw per day for rats and hamsters (Documentation provided to EFSA n. 46), and 3,060 mg calcium carrageenan/kg bw per day for rats (Collins et al., 1977a), the highest doses tested;
- the Panel considered that for the safety evaluation of processed Eucheuma seaweed (E 407a) read across can be made from study results used in the toxicological evaluation of carrageenan (E 407).

In addition, the Panel observed that:

- from all the data received, data were adequate for a refined exposure assessment for 41 out of 79 food categories;
- in the general population, based on the reported use levels, a refined exposure, in the brand-loyal scenario of up to 758.6 mg/kg bw per day for toddlers (from 12 months up to and including 35 months of age) was estimated;
- for populations consuming foods for special medical purposes and special formulae, the 95th percentile of maximum exposure assessments calculated based on the maximum reported data from food industry (equal to the MPL) were up to 49.4 mg/kg bw per day for infants (from 12 weeks up to and including 11 months of age);

For the food additives E 407 and E 407a, the fraction of low molecular weight carrageenan is limited in the EC specifications by the purity criteria. The Panel was informed by one interested party that the material on the market does not necessarily comply with the EU specifications regarding the limit of the low molecular weight fraction. This fraction has been associated with potential health hazards similar to those reported for preparations of degraded carrageenan, such as poligeenan or C16, to which this fraction shows similarity in molecular structure and in weight-average molecular weight. Although, full identity of degraded carrageenan such as poligeenan or C16 with the low molecular weight fraction of carrageenan has not been specifically demonstrated.

Concerning degraded carrageenan (e.g. poligeenan, C16), the Panel therefore noted the following:

- degraded carrageenan has been described to be absorbed and to be present in various tissues, namely the liver, and the urine of animals when administered in drinking water or via the diet;
- degraded carrageenan did not raise concern with respect to genotoxicity;
- rats exposed to degraded ι-carrageenan (weight-average molecular weight of 20–40 kDa) via drinking water, diet or by gavage for up to 24 months developed in first instance colitis, secondary metaplasia and finally tumours (squamous cell carcinomas, adenocarcinomas, adenomas);
- monkeys given degraded ι-carrageenan (C16) via drinking water showed histopathological lesions in the colon which varied from slight mucosal erosions at the low dose (750 mg/kg bw per day) to ulceration associated with inflammatory infiltration of the lamina propria at the high dose (2,900 mg/kg bw per day). In this study, all monkeys on C16 lost blood frequently from the intestinal tract in a dose-related degree and developed some degree of anaemia. A LOAEL of 750 mg degraded ι-carrageenan (C16)/kg bw per day was noted;
- toxicity studies have been mainly conducted with degraded ι-carrageenan, thus, degraded κ- and λ-carrageenan being sparsely toxicologically characterised.

The following uncertainties were noted by the Panel as regards the chemistry and fate of carrageenan (E 407) and processed Eucheuma seaweed (E 407a):

- no data are available showing the molecular weight distribution for different food-grade carrageenan preparations within the defined weight-average molecular weight range of 200–800 kDa;
- no data are available showing the molecular weight distribution for individual food-grade processed Eucheuma seaweed preparations;
- the weight-average molecular weight range of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) is not defined in the EU specifications allowing for the presence of a low weight-average molecular weight fraction of carrageenan. Based on the information provided on weight-average molecular weights of commercially available carrageenans, the low molecular weight material needs to be accurately quantified;
- in most of the toxicological studies the carrageenan used is not well specified and its weight-average molecular weight and its content of low molecular weight fragments are not given;
- although it has been claimed that there is no adequate analytical method available to measure the low molecular weight fraction, the Panel noted that there is indication of the percentage of the low molecular weight fraction in the food additive carrageenan (E 407) tested in a few toxicological studies;
- only limited information on the stability of carrageenan in food was available. No data on stability of carrageenan and/or processed Eucheuma seaweed addressing the usual variation of parameters (temperature, pH) relevant for the authorised food uses were available. Information on possible degradation products under acidic conditions in relevant food products is missing;
- studies investigating the hydrolysis of the κ-, ι- and λ-carrageenan showed contradictory results.

The Panel further noted the following uncertainty as regards the exposure assessment scenario:

- the refined estimates were based on 41 out of 79 food categories in which carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised and result in an overestimation of the real exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives in European countries considering only food additives uses for which data have been provided were considered.

Among the uncertainties from the biological and toxicological data, the Panel considered the following:

- the lack of reliable comparative toxicokinetic and toxicological studies between the different types of carrageenan and their corresponding low molecular weight fractions;
- the theoretical possibility that limited degradation could occur under conditions representative of the *in vivo* situation;
- no firm conclusion on the other types of carrageenan could be drawn on the observation of occult blood in faeces of rhesus monkeys dosed with a commercial carrageenan;
- the characterisation of the test material in most of the toxicological studies was limited;
- there were no adequate toxicological studies performed with low weight-average molecular weight carrageenan (around 200 kDa), apart from one 90-day study (with an average molecular weight carrageenan in the range of 196–257 kDa, not specified if it was a number-average or a weight-average);
- testing for chronic toxicity and reproductive and developmental toxicity was performed almost exclusively with κ/λ-carrageenan; almost no data on ι-carrageenan were available;
- inadequate data on the possible relevance of carrageenan exposure for existing inflammatory bowel diseases in humans;
- the relevance for humans of the observations in animal studies pointing to the induction of glucose intolerance and glucosuria by carrageenan is unclear;
- the possible role of sulfate and the interactions of the various forms of carrageenans with the gut microflora in some of the reported inflammatory effects of carrageenans.

Altogether in most toxicological studies, the molecular weight distribution of the carrageenan tested is not or only inadequately described. The low molecular weight fraction of carrageenan is associated with potential adverse effects. This is due to its similarity in molecular structure and weight-average molecular weight with those of degraded carrageenan, such as poligeenan or C16, known to cause inflammatory intestinal effects. Moreover, results suggesting that carrageenan might enhance inflammatory bowel disease in humans need clarification. The test compounds used in a large number of the toxicity tests are thus not considered to reflect adequately the diversity of preparations of the food additive on the market, particularly with respect to the broad range of weight-average molecular weights reported. The Panel noted that most of these issues were also raised and discussed in a recent review (David et al., 2018).

The Panel also considered other pending uncertainties regarding the relevance of the studies available to assess the safety of the authorised food additive carrageenan (E 407). The physicochemical properties of carrageenan depend on the chemical conformation (helical or random coil) in which it exists in the preparations and in foods (solid or liquids foods) and are influenced by the presence of cations, proteins and the pH and temperature. These conditions could affect the toxicity of carrageenan and are thus relevant for the safety assessment of the food additive in the authorised uses. Furthermore, findings in some studies suggest that carrageenan exposure could be related to the induction of glucose intolerance and glucosuria. In the view of the Panel, all this information still needs clarification.

Overall, taking into account the lack of adequate data to address the above-mentioned uncertainties, the Panel concluded that the existing group ADI for carrageenan (E 407) and processed Eucheuma seaweed (E 407a) of 75 mg/kg bw per day should be considered temporary, while the database should be improved within 5 years after publication of this opinion. Within the given time frame, high importance should be ascribed to the establishment of an adequate interlaboratory validated analytical method to quantify, at the existing 5% limit, the low weight-average molecular weight fraction of carrageenan, and to establish whether or not this fraction is associated with health risks.

The Panel further concluded that in the refined brand-loyal exposure assessment scenario the exposure estimates exceeded, in some cases by up to approximately 10-fold, the temporary existing ADI at the high level (95th percentile) for all population groups and at the mean for all population groups except for infants and the elderly. Although the exposure may be overestimated, the extent of the exceedance of the ADI (10-fold) may be a safety concern.

The Panel recommended that the EC considers:

- in the definition for the food additives E 407 and E 407a in the Commission Regulation (EU) No 231/2012, specifying the weight-average molecular weight range in a narrow way avoiding a significant overlap with the molecular weight range of preparations of degraded carrageenan;

- with respect to the purity criteria in the Commission Regulation (EU) No 231/2012, the need of an interlaboratory validated analytical method to detect low molecular weight carrageenan in the food additives E 407 and E 407a at the limit specified in the Regulation;
- possibility of extending the molecular weight of the existing 5% limit for low molecular weight carrageenan from 50 kDa to up to 200 kDa for the food additives E 407 and E 407a in the Commission Regulation (EU) No 231/2012. This would take into account the limitation of the available analytical methods to quantify the percentage of low weight-average molecular weight carrageenan and will further make certain that the presence of low weight-average molecular weight carrageenan is at a low level; it should be indicated if the 5% limit refers to the food additive as such or on dried basis.
- the need of information on the stability of the food additives E 407 and E 407a in food. No data on stability of carrageenan (E 407) and/or processed Eucheuma seaweed (E 407a) addressing the usual variation of parameters (temperature, pH) relevant for the authorised food uses were available. Information on possible degradation products under acidic conditions in relevant food products was missing;
- revising the maximum limits for the impurities of toxic elements (lead, mercury, cadmium and arsenic) in the EC specification for carrageenan (E 407) and processed Eucheuma seaweed (E 407a) in order to ensure that the food additives will not be a significant source of exposure to these toxic elements in food;
- collecting monitoring data for the food categories contributing the most to the exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) in order to allow for more refined estimates of exposure;
- establishing, as a general principle, numerical MPLs for all food authorised uses with special consideration of the main food categories contributing to the exposure (e.g. flavoured milk products, flavoured drinks, food supplements and fine bakery wares) to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives in order to reduce consumers exposure.

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1. Introduction

The present opinion deals with the re-evaluation of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) when used as food additives.

1.1. Background and Terms of Reference as provided by the European Commission

1.1.1. Background as provided by the European Commission

Regulation (EC) No 1333/2008[2] of the European Parliament and of the Council on food additives requires that food additives are subject to a safety evaluation by the European Food Safety Authority (EFSA) before they are permitted for use in the European Union. In addition, it is foreseen that food additives must be kept under continuous observation and must be re-evaluated by EFSA.

For this purpose, a programme for the re-evaluation of food additives that were already permitted in the European Union before 20 January 2009 has been set up under the Regulation (EU) No 257/2010[3]. This Regulation also foresees that food additives are re-evaluated whenever necessary in light of changing conditions of use and new scientific information. For efficiency and practical purposes, the re-evaluation should, as far as possible, be conducted by group of food additives according to the main functional class to which they belong.

The order of priorities for the re-evaluation of the currently approved food additives should be set on the basis of the following criteria: the time since the last evaluation of a food additive by the Scientific Committee on Food (SCF) or by EFSA, the availability of new scientific evidence, the extent of use of a food additive in food and the human exposure to the food additive taking also into account the outcome of the Report from the Commission on Dietary Food Additive Intake in the EU[4] of 2001. The report 'Food additives in Europe 2000[5] 'submitted by the Nordic Council of Ministers to the Commission, provides additional information for the prioritisation of additives for re-evaluation. As colours were among the first additives to be evaluated, these food additives should be re-evaluated with a highest priority.

In 2003, the Commission already requested EFSA to start a systematic re-evaluation of authorised food additives. However, as a result of adoption of Regulation (EU) 257/2010 the 2003 Terms of References are replaced by those below.

1.1.2. Terms of Reference as provided by the European Commission

The Commission asks the European Food Safety Authority to re-evaluate the safety of food additives already permitted in the Union before 2009 and to issue scientific opinions on these additives, taking especially into account the priorities, procedures and deadlines that are enshrined in the Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with the Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives.

1.1.3. Interpretation of Terms of Reference

The ANS Panel described its risk assessment paradigm in the Guidance for submission for food additive evaluations in 2012 (EFSA ANS Panel, 2012a,b). This Guidance states, that 'in carrying out its risk assessments, the Panel seeks to define a health-based guidance value, e.g. an Acceptable Daily Intake (ADI) (IPCS, 2004) applicable to the general population'. According to the guidance above mentioned, the ADI as established for the general population does not apply to infants below 12 weeks of age (JECFA, 1978; SCF, 1998b). In this context, the re-evaluation of the use of food additives in food for infants below 12 weeks represents a special case for which specific recommendations were given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA)

[2] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16–33.
[3] Commission Regulation (EU) No 257/2010 of 25 March 2010 setting up a programme for the re-evaluation of approved food additives in accordance with Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives. OJ L 80, 26.3.2010, p. 19–27.
[4] COM(2001) 542 final.
[5] Food Additives in Europe 2000, Status of safety assessments of food additives presently permitted in the EU, Nordic Council of Ministers, TemaNord 2002, 560.

(JECFA, 1972, 1978) and by the SCF (SCF, 1996, 1998a) and by EFSA (EFSA Scientific Committee, 2017). The Panel endorsed these recommendations.

In the current EU legislation (Annex II of Regulation (EC) No 1333/2008), use levels of additives in food for infants under the age of 12 weeks are included in categories 13.1.1, 13.1.5.1 and 13.1.5.2.[6] The Panel considers that these uses would require a specific risk assessment in line with the recommendations given by JECFA and the SCF and endorsed by the Panel by the Panel in its current Guidance for submission for food additives evaluations (EFSA ANS Panel, 2012a,b). Therefore, risk assessments for the general population are not considered applicable for infants under the age of 12 weeks and will be performed separately by EFSA.[7]

In the present opinion, the term 'carrageenan' is used synonymously with the terms 'native carrageenan' or 'undegraded carrageenan' or 'conventionally processed carrageenan'.

Poligeenan (CAS No. 53973-98-1) also called 'polygeenan' is a specific type of degraded ι- carrageenan. C16 is another type of degraded ι-carrageenan. The Panel noted that poligeenan and C16 are not authorised food additives and they are not the same as the authorised food additive carrageenan (E 407). The Panel noted that in some studies degraded carrageenan derived from κ- and λ-types has been used.

1.2. Information on existing evaluations and authorisations

Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised as food additives in the EU in accordance with Annex II and Annex III to Regulation (EC) No 1333/2008 on food additives and specific purity criteria have been defined in the Commission Regulation (EU) No 231/2012[1], where in the definition is stated that: 'the wording carrageenan is reserved for the non hydrolysed or otherwise chemically degraded polymer'.

In the EU, carrageenan (E 407) and processed Eucheuma seaweed (E 407a) have been evaluated by the SCF in 1977 (SCF, 1978) and 1996, respectively. The SCF endorsed the ADI of 0–75 mg/kg body weight (bw) per day for carrageenan established by JECFA (1974) without other information. In 1994, the SCF evaluated the possible degradation of carrageenan in food, the uptake of small amounts of carrageenan in certain species, the effect on immune system in animals and the increased intestinal permeability found in very young infants. While the SCF considered that 'there is no direct evidence of harm from carrageenan in human infants and no toxicologically significant effects were seen in infant baboons fed carrageenan in commercial infant formulae', the SCF did not exclude 'the possibility that the absorbed material might affect the immune system in infant' and consequently 'did not consider carrageenan acceptable for use in infant formulae' (SCF, 1994a).

Toxicological data for processed Eucheuma seaweed (E 407a) were evaluated by the SCF in 1996 (opinion on 'alternatively refined carrageenan' produced from *Eucheuma cottonii* and *Eucheuma spinosum*). The Committee considered the 'alternatively refined carrageenan' (processed Eucheuma seaweed (E 407a)) to be an acceptable carrageenan preparation. The absence of genotoxicity and of toxic effects in the 90-day study (no references given by the SCF but presumably the same database was used as by JECFA, 1999) support this opinion of the SCF and its conclusion that further toxicological testing is not needed. The SCF maintained the original group ADI for all types of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) of 0–75 mg/kg bw because of some uncertainty 'about the general immuno-reactive potential of the various carrageenans now in use as food additives' (SCF, 1996).

In the latest opinion dated 2003, the SCF evaluated the following: 'In this review the Committee has addressed several issues. The first issue discussed by Tobacman (2001) was the possibility of exposure to degraded carrageenan. Degraded carrageenan, also called poligeenan,[8] has a weight-average molecular weight of 20–30 kDa (Weiner, 1991). It is known to cause haemorrhage and ulceration of the large intestine in some laboratory animal species (guinea pig, rabbit and monkey), whereas undegraded food-grade carrageenan, with a weight-average molecular weight of above

[6] Food of category 13.1.1: Infant formulae as defined by Directive 2006/141/EC; Food of category 13.1.5.1: Dietary foods for infants for special medical purposes and special formulae for infants. This interpretation also applies to those food additives in food category 13.1.5.2 (Dietary foods for babies and young children for special medical purposes as defined in Directive 1999/21/EC) for which exceptional uses in food for infants under the age of 12 weeks are indicated.

[7] EFSA-Q-2018-00270 Question number of carrageenan and PES.

[8] Poligeenan (CAS No. 53973-98-1) is an artificially formed polymer produced by subjecting carrageenan to extensive acid hydrolysis at low pH (0.9–1.3) and high temperatures (> 80°C) for an extended period of time until the weight-average molecular weight (Mw) is reduced to the range 10–20 kDa. For more information see Section 3.1.1.

100 kDa, does not (SCF, 1978; JECFA, 1984). Tobacman (2001) discussed the possible sources of degraded carrageenan, i.e. contamination by lower molecular weight components in the additive as such, generation of degraded carrageenan during the processing of foods containing the additive, or digestion of the additive by acid hydrolysis or degradation by bacteria in the gut. The second issue raised by Tobacman (2001) was a possible tumour promotion effect of undegraded carrageenan in the gut. In considering these issues, the Committee took into consideration the recent review of carrageenan by JECFA (2002). The other paper of Tobacman (2001) considered in this review proposed an hypothesis that the increasing incidence of breast cancer in the USA during the twentieth century may be related to consumption of carrageenan and possibly other water-soluble polymers. The Committee also reconsidered the unresolved issue of whether carrageenan might be absorbed by the immature gut and have effects on immune function in the very young (SCF, 1994b)'.

The SCF concluded that: 'The Committee concluded that the information available since its last review of carrageenan as an additive for general food use did not provide any reason to alter the ADI of 0–75 mg/kg bw established previously. The Committee notes that intakes are considerably below the ADI. The Committee does however consider that, if feasible, a molecular weight limit of not > 5% below 50 kDa should be introduced into the specification, in order to ensure that the presence of any degraded carrageenan is kept to a minimum. In the absence of any further information on possible absorption of carrageenan by the immature gut in the very young infant, the Committee reaffirms its earlier view (SCF, 1998a) that it remains inadvisable to use carrageenan in infant formulae that are fed from birth, including those in the category of foods for special medical purposes. The Committee has no objection to the use of carrageenan in foods for older infants, such as follow-on milks (SCF, 1983) and weaning foods (SCF, 2003a)'.

In 2003, the SCF also re-evaluated carrageenan in the revision of the essential requirements of infant formulae and follow-on formulae intended for the feeding of infants and young children (SCF, 2003b). The Committee reconfirmed its view that carrageenan should not be used in infant formulae. However, the Committee had no objection to the use of carrageenan in follow-on formulae up to a maximum level of 0.3 g/L. 'The Committee further recommended maintaining the concept that if more than one of the three substances locust bean gum, guar gum or carrageenan are added to a follow-on formula, the maximum level established for each of those substances is lowered with that relative part as is present of the other substances together (SCF, 2003b)'.

Carrageenan (E 407) was also evaluated by JECFA in 1973, 1984, 2000, 2002, 2007, 2008 and 2015 (JECFA, 1974, 1984, 2000, 2002, 2007b, 2008, 2015). JECFA allocated an ADI of 0–75 mg/kg bw (JECFA, 1974), which was changed to an ADI 'not specified' in 1984 (JECFA, 1984) on the basis of a number of toxicological studies on carrageenan from different sources.

Processed Eucheuma seaweed (E 407a) was evaluated by JECFA in 1987, 1992, 1993, 1995, 2000 and 2002 (JECFA, 1987, 1992, 1993, 1995, 2000, 2002). In 2000, JECFA concluded that 'the toxicities of processed Eucheuma seaweed and carrageenan were sufficiently similar for the ADI "not specified" for carrageenan to be extended to a temporary group ADI including processed Eucheuma seaweed, pending clarification of the significance of the tumour promotion of known experimental colon carcinogens by carrageenan observed in experiments in rats'.

In 2002, JECFA re-evaluated the cancer-promoting activity using recent data on carrageenan (JECFA, 2002). In relevant studies, it was shown that the administration of carrageenan at concentrations of up to 5% in the diet did not promote colon carcinogenesis. Therefore, the Committee considered that the intake of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives was of no concern. In this JECFA evaluation, a group ADI 'not specified' was allocated to the sum of carrageenan (E 407) and processed Eucheuma seaweed (E 407a).

In 2007, JECFA reviewed all the available toxicological data, including data related to the safety assessment for infants from exposure via infant formula. JECFA estimated margins of exposure (MOEs) for infants and was of the view that 'it is inadvisable to use carrageenan or processed Eucheuma seaweed in infant formula intended for infants up to and including 12 months of age', because the database was not sufficient to draw conclusions on (1) effects of carrageenan on the immature gut, (2) absorption of carrageenan by the immature gut and (3) effects of carrageenan on the immune response of the gastrointestinal tract (JECFA, 2007b, 2008). Therefore, the group ADI 'not specified' for the sum of carrageenan and processed Eucheuma seaweed was maintained for food additive uses in foods other than infant formula (JECFA, 2007b).

In its latest evaluation in 2015, JECFA reviewed data published since 2007 taking into account new studies relevant to the evaluation of the use of carrageenan in infant formula and formula for special

medical purposes, including studies on absorption and toxicity in both the neonatal minipig and neonatal pig. JECFA also considered other data available in the literature related to carrageenan and to the signalling pathways involved in inflammation. The Committee noted that although the MOEs were small, these were derived from a neonatal pig study in which no adverse effects on the gut or on immune parameters were observed at the highest dose tested. JECFA also took into account the previous toxicological database on carrageenan, which did not indicate other toxicological concerns. Overall, JECFA concluded that 'the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1,000 mg/L is not of concern' (JECFA, 2015).

Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are two of the food additives that composed jelly mini-cups which were suspended in 2004 by the European Commission to be placed on the market and import (Commission Decision 2004/37/EC, European Commission, 2004), following the measures taken and information provided by different Member States. Jelly mini-cups are defined as 'jelly confectionery of a firm consistence, contained in semi rigid mini-cups or mini-capsules, intended to be ingested in a single bite by exerting pressure on the minicups or mini-capsule to project the confectionery into the mouth'.

In 2004, the EFSA Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) prepared a scientific opinion on a request from the European Commission related to the use of certain food additives derived from seaweed or non-seaweed origin, including carrageenan (E 407) and processed Eucheuma seaweed (E 407a) in jelly mini-cups (EFSA AFC Panel, 2004). The AFC Panel concluded that any of these gel-forming additives or of 'any other type that gave rise to a confectionery product of a similar size, with similar physical and/or physicochemical properties and that could be ingested in the same way as the jelly mini-cups, would give rise to a risk for choking' (EFSA AFC Panel, 2004). The use of these additives in jelly mini-cups is not authorised in the EU.[9]

In 2005, the EFSA AFC Panel prepared a scientific opinion on the risks posed by semicarbazide in all types of food. According to EFSA (2005), processed Eucheuma seaweed (E 407a) is sometimes bleached with calcium hypochlorite resulting in higher concentrations of the reaction by-product semicarbazide; industry initiated trials to replace calcium hypochlorite with an alternative bleaching agent for the production of processed Eucheuma seaweed (E 407a). For processed Eucheuma seaweed prepared using a bleaching step, for the 25 batches reported by Marinalg to the AFC Panel the range for semicarbazide was 9–380 μg/kg with a mean of 65 μg/kg. According to the AFC Panel, 'for the food additive carrageenan, that may become contaminated with semicarbazide at a mean concentration of 65 μg/kg from use of hypochlorite in the production process, if consumption was up to the ADI for carrageenan of 75 mg/kg bw per day then the intake of semicarbazide from this source could be up to 0.005 μg/kg bw per day'. Based on the overall weight of evidence, the AFC Panel concluded that 'the weak genotoxicity exerted by semicarbazide *in vitro* is not expressed *in vivo*…. A large margin of at least 5 orders of magnitude exists between the dose causing tumours in experimental animals and human exposure, including that of infants'. Therefore, the AFC Panel concluded that 'the issue of carcinogenicity is not of concern for human health at the concentrations of semicarbazide encountered in food' (EFSA, 2005).

In 2006, the EFSA AFC Panel prepared a scientific opinion on use of formaldehyde as a preservative during the manufacture and preparation of food additives. The Panel estimated that exposure to gelling additives such as carrageenans containing residual formaldehyde at the levels of 50 mg/kg of additive would be of no safety concern (EFSA, 2007). Maximum limits (not more than 50 mg/kg) are established in the current EC Regulation for formaldehyde in several thickening food additives from algae origin including carrageenan (EU No 231/2012).

Regarding the food additive carrageenan (E 407), the International Agency for Research on Cancer (IARC), in 1983 classified carrageenan in group 3 considering that the available data did not provide evidence that carrageenan is carcinogenic to experimental animals, in the absence of human data. IARC also evaluated 'degraded carrageenan' and concluded that 'experiments in rats with doses of degraded carrageenan comparable to those used to test carrageenan provide sufficient evidence for the carcinogenicity of degraded carrageenan in rats. No data on humans were available' (category 2B).

A review of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) was published from the Nordic Council of Ministers. In the report, it was concluded for carrageenan (E 407) that: 'Carrageenan has been extensively studied and in most studies seems to pose no toxicological problem. However the question about the potential promotion of colon carcinogenesis by carrageenan

[9] Annex II to Regulation (EC) No 1333/2008.

should be clarified. SCF has expressed the wish to review carrageenan in the light of this aspect. JECFA, however, at its 57th meeting June 2001, withdrew the temporary status and allocated a full ADI "not specified". It may be necessary to clarify whether all types of carrageenan are sufficiently covered by the toxicological evaluation'. It was further concluded for processed Eucheuma seaweed (PES, E 407a) that: 'Because of the similarities in the nature of processed Eucheuma seaweed and the conventionally processed carrageenans and in the effects they caused in the recent 90-day study it can be concluded that PES can be included in the evaluation of carrageenans. processed Eucheuma seaweed as defined by the specifications is covered by the toxicological evaluation' (TemaNord, 2002).

2. Data and methodologies

2.1. Data

The Panel was not provided with a newly submitted dossier. EFSA launched public calls for data,[10,11,12] to collect relevant information from interested parties.

The Panel based its assessment on information submitted to EFSA following the public calls for data, information from previous evaluations and additional available literature up to the last Working Group meeting before the adoption of the opinion.[13] Attempts were made at retrieving relevant original study reports on which previous evaluations or reviews were based, however, not always these were available to the Panel.

The EFSA Comprehensive European Food Consumption Database (Comprehensive Database[14]) was used to estimate the dietary exposure.

The Mintel's Global New Products Database (GNPD) is an online database which was used for checking the labelling of products containing carrageenan (E 407) and processed Eucheuma seaweed (E 407a) within the EU's food products as GNPD shows the compulsory ingredient information presented in the labelling of products.

2.2. Methodologies

This opinion was formulated following the principles described in the EFSA Guidance on transparency in the scientific aspects of risk assessment (EFSA Scientific Committee, 2009) and following the relevant existing Guidances from the EFSA Scientific Committee.

The ANS Panel assessed the safety of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives in line with the principles laid down in Regulation (EU) 257/2010 and in the relevant guidance document: Guidance on submission for food additive evaluations by the Scientific Committee on Food (SCF, 2001) and taking into consideration the Guidance for submission for food additive evaluations in 2012 (EFSA ANS Panel, 2012a,b).

When the test substance was administered in the feed or in the drinking water, but doses were not explicitly reported by the authors as mg/kg bw per day based on actual feed or water consumption, the daily intake was calculated by the Panel using the relevant default values as indicated in the EFSA Scientific Committee Guidance document (EFSA Scientific Committee, 2012) for studies in rodents or, in the case of other animal species, by JECFA (2000). In these cases, the daily intake is expressed as equivalent. When in human studies in adults (aged above 18 years), the dose of the test substance administered was reported in mg/person per day, the dose in mg/kg bw per day was calculated by the Panel using a body weight of 70 kg as default for the adult population as described in the EFSA Scientific Committee Guidance document (EFSA Scientific Committee, 2012).

Dietary exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives was estimated combining food consumption data available within the EFSA Comprehensive European Food Consumption Database with the maximum permitted levels (MPLs) and/or reported use levels and analytical data submitted to EFSA following a call for data. Different

[10] Call for scientific data on food additives permitted in the EU and belonging to the functional classes of emulsifiers, stabilisers and gelling agents. Published: 22 November 2009. Available from: http://www.efsa.europa.eu/en/dataclosed/call/ans091123

[11] Call for food additives usage level and/or concentration data in food and beverages intended for human consumption - Extended deadline: 30 September 2014 Available online: http://www.efsa.europa.eu/sites/default/files/consultation/140310.pdf

[12] Call for technical data on certain thickening agents permitted as food additives in the EU - Extended Deadline: 31 December 2015. Available online: http://www.efsa.europa.eu/it/data/call/141219

[13] 6/3/2018.

[14] Available online: http://www.efsa.europa.eu/en/datexfoodcdb/datexfooddb.htm

exposure scenarios were calculated (see Section 3.4.1). Uncertainties on the exposure assessment were identified and discussed.

In the context of this re-evaluation, the Panel considered the conceptual framework for the risk assessment of certain food additives re-evaluated under Commission Regulation (EC) No 257/2010 (EFSA, 2014).

3. Assessment

3.1. Technical data

3.1.1. Identity of the substances

3.1.1.1. Carrageenan (E 407)

According to the Commission Regulation (EU) No 231/2012[1], 'carrageenan (E 407) is obtained by extraction with water or dilute aqueous alkali of strains of seaweeds of *Gigartinaceae*, *Solieriaceae*, *Hypneaceae* and *Furcellariaceae*, families of the class *Rhodophyceae* (red seaweeds). It consists chiefly of the potassium, sodium, magnesium and calcium sulphate esters of galactose and 3,6-anhydrogalactose polysaccharides. These hexoses are alternately linked α-1,3 and β-1,4 in the copolymer. The prevalent polysaccharides in carrageenan are designated as κ, ι, λ depending on the number of sulphate by repeating unit (i.e. 1,2,3 sulphate). Between κ and ι there is a continuum of intermediate compositions differing in number of sulphates per repeat units between 1 and 2. During the process, no organic precipitant shall be used other than methanol, ethanol and propan-2-ol. The wording "carrageenan" is reserved for the non-hydrolysed or otherwise chemically degraded polymer. Formaldehyde may be present as an adventitious impurity up to a maximum of 5 mg/kg'.

Three major types of carrageenan can be distinguished by the number and position of the sulfate groups on the disaccharide repeating unit (Marburger, 2003; Draeger et al., 2011):

1) κ-carrageenan with one sulfate group at C4 of the β-D-galactose,
2) ι-carrageenan with one sulfate group at C4 of the β-D-galactose and one sulfate group at C2 of the α-3,6-anhydro-D-galactose, and
3) λ-carrageenan with one sulfate group at C2 of the β-D-galactose and two sulfate groups at C2 and C6 of the α-D-galactose.

The names κ-, ι- and λ-carrageenan do not reflect definitive chemical structures but only general differences in the composition and degree of sulfation at specific locations in the polymer (JECFA, 2001).

The structural formulae of the major structure units are presented in Figure 1.

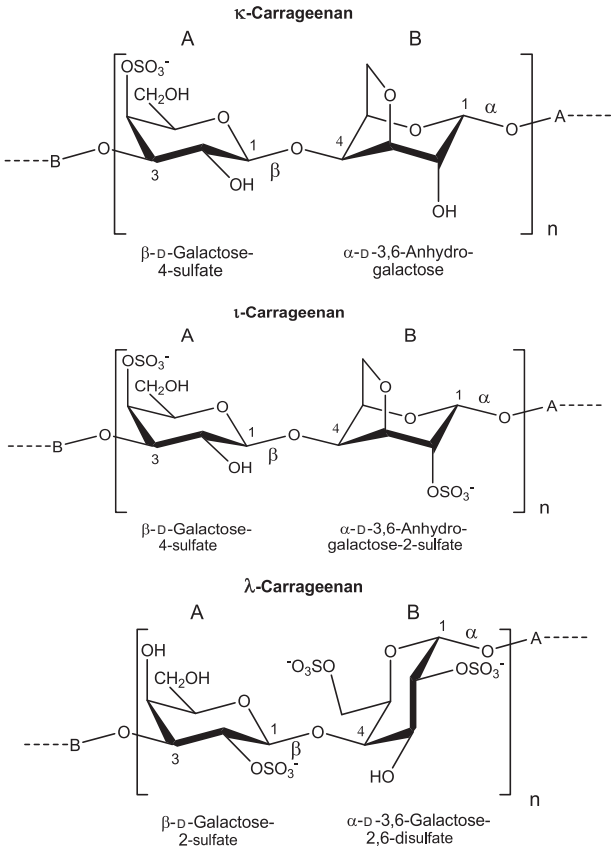


Figure 1: Structural formula of κ-, ι- and λ-carrageenan

In addition to sulfate, substitution by methyl ethers and pyruvate or sugars, such as xylose, glucose and uronic acid, may be present in minor amounts (Voragen et al., 2007). According to JECFA (2014), 'articles of commerce may include sugars for standardisation purposes, salts to obtain specific gelling or thickening characteristics, or emulsifiers carried over from drum drying processes'.

The chemical name of carrageenan is sulfate esters of polygalactose (Commission Regulation (EU) No 231/2012).

It appears as a yellowish to colourless, coarse to fine powder which is practically odourless (Commission Regulation (EU) No 231/2012; JECFA, 2014). The EINECS number for carrageenan is 232-524-2, for κ-carrageenan is 234-350-2, for ι-carrageenan is 232-949-3 and for λ-carrageenan is 232-953-5. CAS number for carrageenan is 9000-07-1, 11114-20-8 for κ-carrageenan, 9062-07-1 for ι-carrageenan and 9064-57-7 for λ-carrageenan.

Carrageenan is usually considered as water soluble, the solubility depends on the type of carrageenan, temperature, pH and the counter ion. While λ-carrageenan is readily water-soluble, κ-carrageenan is the most difficult to dissolve. The sodium salt of κ-carrageenan is soluble in cold water, whereas the potassium salt is soluble only by heating. ι-carrageenan has an intermediate solubility (van de Velde and De Ruiter, 2002). All types of carrageenan are insoluble in organic solvents including alcohols and ketones (Marburger, 2003). For additional information on solubility, see the EU and JECFA specifications (Section 3.1.2).

According to Commission Regulation (EU) No 231/2012, carrageenan is known under several synonyms such as Irish moss gelose (only synonym for *Chondrus*-derived carrageenan), Eucheuman (from *Eucheuma* spp.), Iridophycan (from *Iridaea* spp.), Hypnean (from *Hypnea* spp.), Furcellaran or Danish agar (from *Furcellaria fastigiata).* Other references gave the synonym carrageenin for carrageenan (Merck Index, 2006; Martindale, 2014).

Over time, the sources for the production of carrageenan have changed as have also for certain species their names (see Appendix I). Thus, the original source was *Chondrus crispus*, which still is used to a limited extend, while today most carrageenan is produced from the two sources *Eucheuma cottonii (synonym: Kappaphycus alvarezii)* and *Eucheuma spinosum (synonym: Eucheuma denticulatum*) (FAO, 2003).

Different seaweeds produce different types of carrageenan, the composition being strongly dependent on the algal species (Marburger, 2003; Draeger et al., 2011; FAO, 2003, Table 1).

Table 1: The carrageenan composition in selected red seaweeds (FAO, 2003[(a)])

Chondrus crispus	Mixture of κ and λ
Eucheuma cottonii	Mainly κ
Eucheuma spinosum	Mainly ι
Gigartina skottsbergii	Mainly κ, some λ
Sarcothalia crispata	Mixture of κ and λ

(a): FAO, 2003 (also available on: http://www.fao.org/docrep/006/y4765e/y4765e0a.htm)

One of the interested parties (Documentation provided to EFSA n. 17) provided a list of representative seaweed species with their carrageenan components used in the production of carrageenan and processed Eucheuma seaweed (Table 2).

Table 2: List of representative seaweed species with their carrageenan components

Seaweed species	Extract type	Carrageenan components
Chondrus crispus	E 407	κ/λ[(a)]
Chondrus ocellatus	E 407	κ/λ*
Gigartina stellata	E 407	κ/λ*
Gigartina pistillata	E 407	κ/λ*
Gigartina acicularis	E 407	κ/λ*
Gigartina radula	E 407	κ/λ*
Eucheuma cottonii	E 407/E 407a	κ
Furcellaria fastigiata	E 407	κ
Hypnea musiformis	E 407	κ
Eucheuma spinosum	E 407/E 407a	ι

(a): Ratio of κ:λ varies by season and location from about 90:10 to about 60:40 with a typical average of about 75:25.

According to one of the interested parties (Documentation provided to EFSA n. 19), carrageenan is:

- 'comprised of a mixture of polysaccharides ranging in size from 30 kDa to as high as 5,000 kDa';
- 'a polydisperse molecule and as such is always discussed in terms of its weight-average-molecular weight';
- 'defined as having a weight-average-molecular weight of 200–800 kDa'.

The Panel noted that in the literature the information on the molecular weight of an individual carrageenan preparation is often unprecise mixing the terms 'molecular weight', 'weight-average molecular weight' and 'number-average molecular weight' which from a chemical point of view are not synonymous.[15] In this opinion, the term describing the molecular weight of the carrageenan preparation tested is used as reported by the authors of the studies.

According to Uno et al. (2001a), a survey of 29 samples of food-grade carrageenan representing κ-, ι- and λ-carrageenan determined a number-average molecular weight of 193–324 kDa and a weight-average molecular weight of 453–652 kDa. The molecular weight distributions of 29 samples of food-grade purified carrageenan were studied by the combined gel permeation/inductively coupled plasma (GPC/ICP) method. According to the authors, 'no obvious peak of poligeenan[16] was detected'. According to Uno et al. (2001a), the 'detection limit' for poligeenan was about 5% in the sample of carrageenan.

The percentage below 50 kDa was referred to by various authors as low molecular-weight tail (LMT) and quantification of the LMT remains difficult and inaccurate by all currently available

[15] According to http://polymerdatabase.com/polymer%20physics/Molecular%20Weight.html, "A polymeric material, such as carrageenan, cannot be characterized by a single molecular weight like an ordinary substance. Instead, a statistical average calculated from the molecular weight distribution has to be used". "The weight-average molecular weight is a good measure for the expected statistical size of the polymer, whereas the number-average molecular weight is a measure for the chain length".

[16] Authors use the term poligeenan for degraded carrageenan with molecular weight: 20–30 kDa.

conventional analytical procedures (Blakemore et al., 2014; Documentation provided to EFSA n. 12; Documentation provided to EFSA n. 18). According to information from one of the interested parties (Documentation provided to EFSA n. 19), 'in native carrageenan, this LMT region represents less than 10% of the total carrageenan molecule'.

In this EFSA opinion, the term 'low molecular-weight tail' is used to describe the fraction of low weight-average molecular weight carrageenan also referring to other cut off values higher than 50 kDa.

Poligeenan, chemical name 3,6-anhydro-4-*O*-β-D-galactopyranosyl-α-D-galactopyranose-2,4′-bis (potassium/sodium sulfate)-(1-3′)-polysaccharide (CAS No. 53973-98-1) is a non-naturally occurring polymer produced by subjecting carrageenan to extensive acid hydrolysis at low pH (0.9–1.3) and high temperatures (> 80°C) for an extended period of time until the weight-average molecular weight is reduced to the range 10–20 kDa (Cohen and Ito, 2006; McKim, 2014; JECFA 2015; USDA 2016). According to Scifinder,[17] poligeenan is produced from ι-carrageenan. It was developed in the 1960s to treat pain associated with peptic ulcers, and its only application today is as a component of x-ray imaging diagnostic products (Watson, 2008; USDA, 2016). The name 'poligeenan' was confirmed in 1988 by the United States Adopted Names (USAN) Council (USAN, 1998). Prior to 1988, 'poligeenan' was referred to as 'degraded carrageenan' in the scientific literature. The terms 'poligeenan' and 'degraded carrageenan' were often used interchangeably in research articles and reports. It has been used in research studies as a surrogate for the low molecular weight fraction of carrageenan (JECFA, 2015; Weiner et al., 2015). Poligeenan is distinct from food-grade carrageenan, and according to the EC specifications the term carrageenan is reserved only for the non-hydrolysed or otherwise chemically degraded polymer. According to information gathered for this opinion, 'although the average molecular weights of carrageenan and poligeenan are significantly different, when the molecular weight distributions of the two substances are compared, there is a small overlap between the two at the lower molecular weight portion of the carrageenan distribution and the higher molecular weight portion of the poligeenan distribution, with the range of interest being between 20 and 50 kDa' (Figure 2) (JECFA, 2015). From Figure 2, the Panel noted that based on the data provided by the industry (Documentation provided to EFSA n. 18) the overlapping between the low weight-average molecular weight tail of carrageenan and the high weight-average molecular weight tail of poligeenan is between approximately 30 kDa to up to approximately 200 kDa, respectively.

The Panel also noted that poligeenan is a non-naturally occurring breakdown product of ι-carrageenan consisting of the same sulfated galactose and sulfated anydrogalactose units, linked by α-1,3- and β-1,4-glycosidic bonds as in ι-carrageenan (see Figure 1). Therefore, the Panel considered that the molecular backbone of poligeenan and of carrageenan do not differ. According to Figure 2 (Documentation provided to EFSA n. 18), poligeenan comprises polysaccharide molecules of molecular weight ranging from approximately 2 to 200 kDa, and the weight-average molecular weight of poligeenan ranges from 10 to 20 kDa. Based on the 'nearly identical molecular structure as carrageenan' and 'similar weight-average molecular weight' poligeenan has been used in experimental studies, as a surrogate for the LMT of carrageenan (Weiner et al., 2015).

[17] Available on: https://www.cas.org/products/scifinder

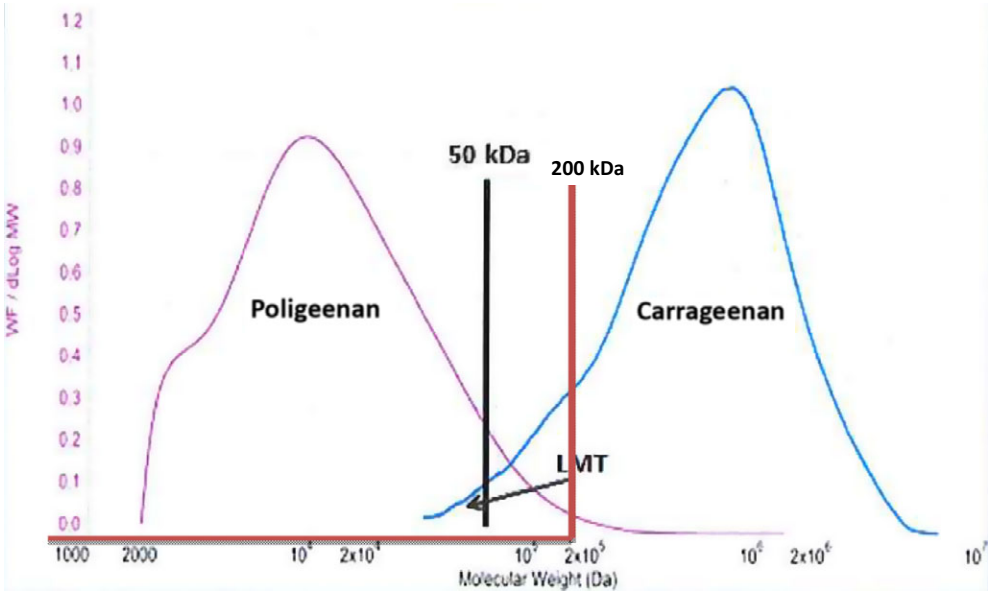


Figure 2: Examples of molecular weight profiles of poligeenan and carrageenan preparations analysed by size exclusion chromatography (SEC) and light scattering (LS). For clarity, the 200 kDa point of reference has been added. For the presented preparation of carrageenan, the weight-average molecular weight is approximately 770 kDa (Documentation provided to EFSA n. 18 and 19)

The Panel noted that no raw data supporting the molecular weight distributions as presented in Figure 2 were provided and that the provided profile is not representative for all kinds of carrageenan products (E 407) on the market.

C16 is a commercially available degraded ι-carrageenan produced by mild acid hydrolysis (prepared from *E. spinosum*, Glaxo Laboratories, France, whereas native ι-carrageenan was a commercial product from Societe Auby, France), reported to have a weight-average molecular weight of 20–30 kDa (Anderson and Soman, 1966) and a number-average molecular weight of 16–19 kDa, (Blakemore and Dewar, 1970).

Recent work has demonstrated that κ-carrageenan also may be acid hydrolysed, using milder conditions (0.1 M H_2SO_4 at 60°C for 1.5 h), to yield exclusively odd-numbered oligosaccharides (Yang et al., 2009). Products from such treatments and from other kinds of mild treatments for degradation of κ-carrageenan have been investigated for their ability to, e.g. protect from recrystallisation of ice cream (Kaminska-Dworznicha et al., 2015a; Kaminska-Dworznicka et al., 2015b, 2016). The authors proposed the designation 'new poligeenan'[18] for such products (Kaminska-Dworznicha et al., 2015a).

For powder commercial carrageenan samples, one of the interested parties (Documentation provided to EFSA n. 16) provided data on mean particle size and distribution for regular mesh (100 mesh) and fine mesh (270 μm mesh) using laser diffraction technology. The smallest particles detected in all samples were 300 nm. Furthermore data were also provided on particle size and distribution of commercial carrageenan product from the two production lines using laser diffraction technology (Documentation provided to EFSA n. 16).

According to the interested party, when used in food applications, all carrageenan powder particles are either dissolved completely into solution, making mean particle size powder data meaningless with respect to the nanoscale, or hydrated with water to swollen particles that are at least ten times greater in size than the powder particles, which takes their mean particle size even further away from the nano-scale (Documentation provided to EFSA n. 16).

Based on the information provided, the Panel noted that nanosized particles (with one or more dimensions below 100 nm) are not present in the powder commercial carrageenan used as a food additive.

Aqueous solutions of carrageenan are highly viscous. Viscosity increases with concentration and the molecular weight, and decreases with temperature. Commercial carrageenan is available in viscosities

[18] No toxicological studies have been identified with this product.

ranging from about 5 mPa·s to 800 mPa·s when measured at a concentration of 1.5% and 75°C (Stanley, 1987; van de Velde and De Ruiter, 2002). Gels are formed by cooling hot solutions of κ- or ι-carrageenan, in the presence of potassium or calcium cations, gelation is accompanied with formation of helices. κ-Carrageenan forms strong, rigid gels with potassium salts and brittle gels with calcium salts. ι-Carrageenan forms elastic gels with calcium salts, while λ-carrageenan has no gel formation ability with these ions (van de Velde and De Ruiter, 2002; Marburger, 2003; Voragen et al., 2007; Draeger et al., 2011; JECFA, 2015; FAO, 2003). In contrary, Running et al. (2012) reported gelation of λ-carrageenan in the presence of trivalent iron ions. The pH values of aqueous solutions are 6.52 (κ-carrageenan), 5.34 (ι-carrageenan) and 5.67 (λ-carrageenan) (Yaseen et al., 2004).

Data on infrared (IR) and raman spectra are presented for carrageenan by European Phamacopoeia (2011); Pereira et al. (2003); van de Velde and De Ruiter (2002). Data on ^{1}H- and ^{13}C-NMR spectra were reported for carrageenan by Bhattacharjee et al. (1978), Pereira et al. (2003) and van de Velde and De Ruiter (2002). Ackloo et al. (2001) reported data on matrix-assisted laser desorption/ionisation and electrospray ionisation mass spectra.

Carrageenan shows physicochemical synergistic interaction with other hydrocolloids, such as xanthan gum, locust bean gum and agar, resulting in an increase in viscosity or gel strength and leading to the ability to impart a desirable elastic character and to retard syneresis in these gels (Klose and Glicksman, 1990; Copetti et al., 1997; Barak and Mudgil, 2014). The synergistic effects occurring when combining carrageenan with locust bean gum, agar or xanthan gum are technologically utilised in food production (Wielinga, 2010).

When carrageenan is present in water at less than approximately 0.1% with no dietary solids or protein, the carrageenan molecules are in disorganised random conformation and available for maximum interaction with other molecules. When carrageenan is dissolved in water at higher concentrations, the carrageenan molecules may be in the disordered (random) or in the ordered (helical) conformation, depending on the carrageenan type and cations present. Only for κ-carrageenan, these helices will aggregate to form a more tightly closed structure. λ-Carrageenan is non-gelling at all concentrations and cation balances, being therefore always in the disorganised conformation in water. Normally λ-carrageenan is present as a minor component in combination with κ-carrageenan in commercial products and increases the gelling matrix through physical (void filling) and chemical (hydrogen-bonded helix cross-linkages) means. The gelation of κ- and ι-carrageenan requires heating to about > 60°C to dissolve the carrageenan at which temperature the carrageenan is in a random conformation (open structure). On cooling to about < 35°C, these carrageenan solutions will gel, first forming double helices (closed structure), and secondly, for κ-carrageenan, these helices will aggregate, as shown in Figure 3 (Blakemore and Harpel, 2010; FMC Corporation 2013; Blakemore et al., 2014; JECFA, 2015).

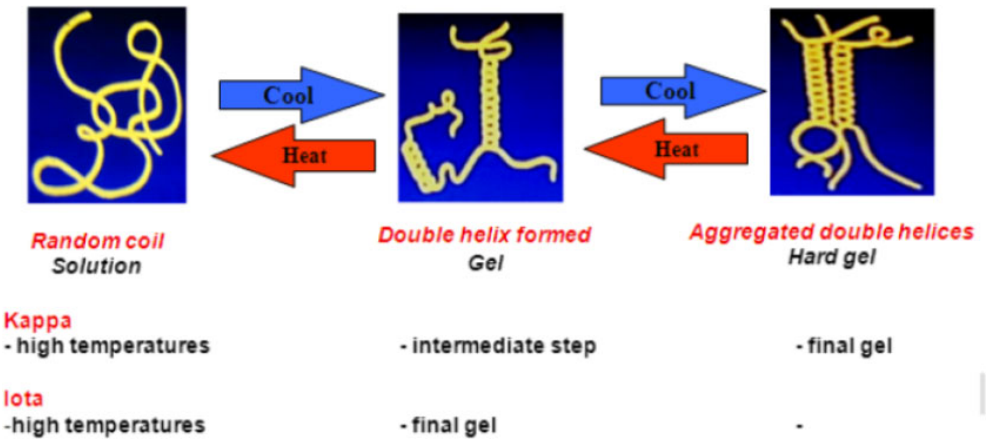


Figure 3: Gelation Mechanism of Carrageenan (re-produced from Blakemore and Harpel, 2010; Documentation provided to EFSA n.18)

Overall the Panel noted that based on the weight-average molecular weight degraded carrageenan, such as poligeenan or C16, is not an authorised food additive and it is different from the authorised food additive carrageenan (E 407). However, in experimental studies, poligeenan has been used as a surrogate for the LMT of carrageenan, based on the similarity of molecular structure and weight-

average molecular weight (Weiner et al., 2015[19]), and therefore, studies with degraded carrageenan are also described in this opinion. It has been confirmed by industry (Documentation provided to EFSA n. 19), that a commercial carrageenan (E 407) may have a weight-average molecular weight of 200 kDa. In view of the Panel, the molecular weight distribution of such a carrageenan product may have a considerable fraction of molecules with a weight-average molecular weight lower than 50 kDa (see section 3.6).

3.1.1.2. Processed Eucheuma seaweed (E 407a)

Processed Eucheuma seaweed (E 407a) is, according to the Commission Regulation (EU) No 231/2012, 'obtained by aqueous alkaline (KOH) treatment at high temperature of the strains of seaweeds *Eucheuma cottonii* and *Eucheuma spinosum*, of the class *Rhodophyceae* (red seaweeds) followed by fresh water washing to remove impurities and drying to obtain the product. Further purification may be achieved by washing with an alcohol. The alcohols authorised are restricted to methanol, ethanol or propane-2-ol. The product consists chiefly of the potassium, sodium, magnesium and calcium sulphate esters of galactose and 3,6-anhydrogalactose polysaccharide. Up to 15% algal cellulose is also present in the product. The wording "processed eucheuma seaweed" is reserved to the non- hydrolysed or otherwise chemically degraded polymer. Formaldehyde may be present up to a maximum of 5 mg/kg'. Neither CAS No nor EINECS No has been assigned to processed Eucheuma seaweed (407a).

Based on structural evaluation, processed Eucheuma seaweed (407a) and carrageenan (E 407) are closely related. Both substances are extracted from red seaweeds (Rhodophyceae), and the main component of processes Eucheuma seaweed (407a) is carrageenan (Marinalg, 2006; Blakemore and Harpel, 2010). Processed Eucheuma seaweed (407a) is distinguished from carrageenan (E 407) by its higher content of cellulosic matter (15%). In addition, the manufacturing does not include solubilisation and precipitation during processing (JECFA, 2007a).

Processed Eucheuma seaweed (407a) is produced from *E. cottonii* which is primarily containing κ-, while the carrageen content from *E. spinosum* is primarily ι- (Marinalg, 2006; JECFA, 2007a).

Processed Eucheuma seaweed (E 407a) is known under several synonyms such as Philippine natural grade (PNG) carrageenan, PES and semi-refined carrageenan (SRC), alternatively refined carrageenan (ARC) (Blakemore and Harpel, 2010).

Processed Eucheuma seaweed (E 407a) appears as tan to yellowish, coarse to fine powder, which is practically odourless (Commission Regulation 231/2012; JECFA, 2007a).

The physicochemical properties of processed Eucheuma seaweed (E 407a) are mainly dependent on its carrageenan content (Marinalg, 2006).

Processed Eucheuma seaweed (E 407a) forms cloudy viscous suspensions in water, it is insoluble in ethanol for a 1.5% solution (Commission Regulation 231/2012). According to JECFA (2007a), '1 g sample disperses and partially dissolves in 100 ml of water at 80°C giving a cloudy opalescent solution. The sample disperses in water more readily if first moistened with alcohol, glycerol, or a saturated solution of glucose or sucrose in water'.

The Panel noted that no raw data showing the molecular weight distribution of processed Eucheuma seaweed (E 407a) were provided.

3.1.2. Specifications

Carrageenan (E 407)

The specifications for carrageenan (E 407) as defined in the Commission Regulation (EU) No 231/2012 and by JECFA (2014) are listed in Table 3.

[19] As reported from Weiner et al. (2015): 'Since PGN, a low Mw polygalactan (Mw ~ 20 kDa), prepared from CGN by severe acid hydrolysis at elevated temperatures, and with a nearly identical molecular structure as CGN, and similar Mw as the LMT, does not bind as strongly to plasma proteins and can be quantitatively extracted from plasma, it is an appropriate and ideal surrogate for the detection of the LMT of CGN for plasma toxicokinetic analysis (Blakemore et al., 2014)' (CGN, carrageenan; Mw, weight-average molecular weight; PGN, poligeenan).

Table 3: Specifications for carrageenan (E 407) according to Commission Regulation (EU) No 231/2012 and JECFA (2014)

	Commission Regulation (EU) No 231/2012	**JECFA (2014)**
Synonyms	Products of commerce are sold under different names such as: Irish moss gelose; Eucheuman (from *Eucheuma* spp.); Iridophycan (from *Iridaea* spp.); Hypnean (from *Hypnea* spp.); Furcellaran or Danish agar (from *Furcellaria fastigiata*); Carrageenan (from *Chondrus* and *Gigartina* spp.)	Irish moss gelose (from *Chondrus* spp.); Eucheuman (from *Eucheuma* spp.); Iridophycan (from *Iridaea* spp.); Hypnean (from *Hypnea* spp.); Furcellaran or Danish agar (from *Furcellaria fastigiata*); INS No. 407
Definition	Carrageenan is obtained by extraction with water or dilute aqueous alkali of strains of seaweeds of Gigartinaceae, Solieriaceae, Hypneaceae and Furcellariaceae, families of the class Rhodophyceae (red seaweeds). Carrageenan consists chiefly of the potassium, sodium, magnesium and calcium sulfate esters of galactose and 3,6-anhydrogalactose polysaccharide. These hexoses are alternately linked α-1,3- and β-1,4 in the copolymer. The prevalent polysaccharides in carrageenan are designated as κ, ι, λ depending on the number of sulfate by repeating unit (i.e. 1,2,3 sulfate). Between κ and ι there is a continuum of intermediate compositions differing in number of sulfates per repeat units between 1 and 2. During the process, no organic precipitant shall be used other than methanol, ethanol and propan-2-ol. The wording carrageenan is reserved for the non hydrolysed or otherwise chemically degraded polymer. Formaldehyde may be present as an adventitious impurity up to a maximum of 5 mg/kg	A substance with hydrocolloid properties obtained from certain members of the class Rhodophyceae (red seaweeds). The principal commercial sources of carrageenans are the following families and genera of the class of Rhodophyceae: Furcellariaceae such as *Furcellaria* Gigartinaceae such as *Chondrus*, *Gigartina*, *Iridaea* Hypneaceae such as *Hypnea* Phyllophoraceae such as *Phyllophora*, *Gynmogongrus*, *Ahnfeltia* Solieriaceae such as *Eucheuma*, *Anatheca*, *Meristotheca* Carrageenan is a hydrocolloid consisting mainly of the ammonium, calcium, magnesium, potassium and sodium sulfate esters of galactose and 3,6-anhydrogalactose polysaccharides. These hexoses are alternately linked α-1,3 and β-1,4 in the copolymer. The relative proportions of cations existing in carrageenan may be changed during processing to the extent that one may become predominant. The prevalent polysaccharides in carrageenan are designated as κ-, ι- and λ-carrageenan. κ-carrageenan is mostly the alternating polymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose; ι-carrageenan is similar, except that the 3,6-anhydrogalactose is sulfated at carbon 2. Between κ-carrageenan and ι-carrageenan there is a continuum of intermediate compositions differing in degree of sulfation at carbon 2. In λ-carrageenan, the alternating monomeric units are mostly D-galactose-2-sulfate (1,3-linked) and D-galactose-2,6-disulfate (1,4-linked). Carrageenan is obtained by extraction from seaweed into water or aqueous dilute alkali. Carrageenan may be recovered by alcohol precipitation, by drum drying, or by precipitation in aqueous potassium chloride and subsequent freezing. The alcohols used during recovery and purification are restricted to methanol, ethanol, and isopropanol. Articles of commerce may include sugars for standardisation purposes, salts to obtain specific gelling or thickening characteristics, or emulsifiers carried over from drum drying processes

	Commission Regulation (EU) No 231/2012	JECFA (2014)
Description	Yellowish to colourless, coarse to fine powder which is practically odourless	Yellowish or tan to white, coarse to fine powder that is practically odourless
EINECS	232-524-2	–
CAS number	–	9000-07-1
Chemical name	Sulfate esters of polygalactose	–
Functional uses	–	Thickener, gelling agent, stabiliser, glazing agent
Identification		
Test for galactose and anhydrogalactose	Passes test	Proceed as directed in Vol. 4 (under 'General Methods, Organic Components, Gum Constituents Identification') using the following as reference standards: galactose, rhamnose, galacturonic acid, 3,6-anhydrogalactose, mannose, arabinose and xylose. Galactose and 3,6-anhydrogalactose should be present
Test for sulfate	Passes test	Dissolve a 100 mg sample in 20 mL of water (with heating if necessary), and add 3 mL of barium chloride TS and 5 mL of hydrochloric acid, dilute TS; filter if a precipitate forms. Boil the solution or the filtrate for 5 min. A white, crystalline precipitate appears
Solubility	Soluble in hot water; insoluble in alcohol for a 1.5% dilution	Insoluble in ethanol; soluble in water at a temperature of about 80°, forming a viscous clear or slightly opalescent solution that flows readily; disperses in water more readily if first moistened with alcohol, glycerol, or a saturated solution of glucose or sucrose in water
Identification of hydrocolloid and predominant type of copolymer	–	Add 4 g of sample to 200 mL of water, and heat the mixture in a water bath at 80°, with constant stirring, until dissolved. Replace any water lost by evaporation, and allow the solution to cool to room temperature. It becomes viscous and may form a gel. To 50 mL of the solution or gel add 200 mg of potassium chloride, then reheat, mix well, and cool. A short-textured ('brittle') gel indicates a carrageenan of a predominantly κ-type, and a compliant ('elastic') gel indicates a predominantly ι-type. If the solution does not gel, the carrageenan is of a predominantly λ-type
Infrared absorption	–	Passes test
Purity		
Solvent residues	Not more than 0.1% of methanol, ethanol, propan-2-ol, singly or in combination	Not more than 0.1% of ethanol, isopropanol, or methanol, singly or in combination
Viscosity	Not less than 5 mPa s (1.5% solution at 75°C)	Not less than 5 cp at 75° (1.5% solution)
Loss on drying	Not more than 12% (105°C, 4 h)	Not more than 12% (105° to constant weight)
pH	–	Between 8 and 11 (1 in 100 suspension)
Sulfates	Not less than 15% and not more than 40% on the dried basis (as SO_4)	Not less than 15% and not more than 40% (as SO_4^{2-}) on the dried basis
Ash	Not less than 15% and not more than 40% determined on the dried basis at 550°C	Not less than 15% and not more than 40% on the dried basis

	Commission Regulation (EU) No 231/2012	JECFA (2014)
Acid-insoluble ash	Not more than 1% on the dried basis (insoluble in 10% hydro-chloric acid)	Not more than 1% Use the ash from the Total ash test
Acid-insoluble matter	Not more than 2% on the dried basis (insoluble in 1% v/v sulfuric acid)	Not more than 2% Use 2 g of sample obtained from part (a) of the procedure for sulfate determination
Low molecular weight carrageenan (Molecular weight fraction below 50 kDa)	Not more than 5%	–
Arsenic	Not more than 3 mg/kg	Not more than 3 mg/kg Determine using an AAS (hydride generation technique) appropriate to the specified level. The selection of sample size and method of sample preparation may be based on principles of methods described in Volume 4 (under 'General Methods, Metallic Impurities')
Lead	Not more than 5 mg/kg	Not more than 5 mg/kg Determine using an AAS (electrothermal atomisation technique) appropriate to the specified level. The selection of sample size and method of sample preparation may be based on principles of methods described in Volume 4 (under 'General Methods, Metallic Impurities').
Mercury	Not more than 1 mg/kg	Not more than 1 mg/kg Determine using AAS (cold vapour generation technique). The selection of sample size and method of sample preparation may be based on principles of methods described in Volume 4 (under 'General Methods, Metallic Impurities').
Cadmium	Not more than 2 mg/kg	Not more than 2 mg/kg Determine using an AAS/(electrothermal atomisation technique) appropriate to the specified level. The selection of sample size and method of sample preparation may be based on principles of methods described in Volume 4 (under 'General Methods, Metallic Impurities').
Microbiological criteria		Initially prepare a 10^{-1} dilution by adding a 50 g sample to 450 mL of Butterfield's phosphate-buffered dilution water and homogenising the mixture in a high-speed blender
Total plate count.	Not more than 5,000 colonies per gram	Not more than 5,000 CFU/g
Yeast and moulds	Not more than 300 colonies per gram	–
Escherichia coli	Absent in 5 g	Negative in 1 g
Salmonella spp.	Absent in 10 g	Negative per test

Processed Eucheuma seaweed (E 407a)

Specifications of processed Eucheuma seaweed (E 407a) have been defined in Commission Regulation (EU) No 231/1012 and by JECFA (2007a). The available specifications are listed in Table 4.

Table 4: Specifications of processed Eucheuma seaweed (E 407a) according to Commission Regulation (EU) No 231/2012 and JECFA (2007a)

	Commission Regulation (EU) No 231/2012 (E 407a)	**JECFA (2007a) (INS 407a)**
Synonyms	PES (acronym for processed Eucheuma seaweed). The processed Eucheuma seaweed obtained from *Eucheuma cottonii* is generally called κ-processed Eucheuma seaweed and the processed Eucheuma seaweed from *Eucheuma spinosum* ι-PES	PES, PNG-carrageenan, semi-refined carrageenan; INS No. 407a
Definition	Processed Eucheuma seaweed is obtained by aqueous alkaline (KOH) treatment at high temperature of the strains of seaweeds *E. cottonii* and *E. spinosum*, of the class Rhodophyceae (red seaweeds) followed by fresh water washing to remove impurities and drying to obtain the product. Further purification may be achieved by washing with an alcohol. The alcohols authorised are restricted to methanol, ethanol or propan-2-ol. The product consists chiefly of the potassium, sodium, magnesium and calcium sulfate esters of galactose and 3,6-anhydrogalactose polysaccharide. Up to 15% algal cellulose is also present in the product. The wording processed Eucheuma seaweed is reserved to the non hydrolysed or otherwise chemically degraded polymer. Formaldehyde may be present up to a maximum of 5 mg/kg	A substance with hydrocolloid properties obtained from either *E. cottonii* or *E. spinosum* (from the *Rhodophyceae* class of red seaweeds). In addition to carrageenan polysaccharides, processed Eucheuma seaweed may contain up to 15% of insoluble algal cellulose and minor amounts of other insoluble matter. Articles of commerce may include sugars for standardisation purposes or salts to obtain specific gelling or thickening characteristics. It is distinguished from carrageenan (INS No. 407) by its higher content of cellulosic matter and by the fact that it is not solubilised and precipitated during processing. The functional component of the product obtained from *E. cottonii* is κ-carrageenan (a copolymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose). From *E. spinosum* it is ι-carrageenan (a copolymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose-2-sulfate). Processing consists of soaking the cleaned seaweed in alkali for a short time at elevated temperatures. The material is then thoroughly washed with water to remove residual salts followed by purification, drying, and milling to a powder. Alcohols that may be used during purification are restricted to methanol, ethanol, and isopropanol
Description	Tan to yellowish, coarse to fine powder which is practically odourless	Light tan to white coarse to fine powder
Identification		
Tests for galactose and anhydrogalactose	Passes test	Proceed as directed in Volume 4 (under 'General Methods, Organic Components, Gum Constituents Identification') using the following as reference standards: galactose, rhamnose, galacturonic acid, 3,6-anhydrogalactose, mannose, arabinose and xylose. Galactose and 3,6-anhydrogalactose should be present
Test for sulfate	Passes test	Dissolve a 100 mg sample in 20 mL of water. Heat to boiling, cool to room temperature, and add 3 mL of barium chloride TS and 5 mL of hydrochloric acid, dilute TS. Filter the mixture. Boil the filtrate for 5 min. A white, crystalline precipitate appears

	Commission Regulation (EU) No 231/2012 (E 407a)	JECFA (2007a) (INS 407a)
Solubility	Forms cloudy viscous suspensions in water. Insoluble in ethanol for a 1.5% solution	Forms cloudy viscous suspensions in water; insoluble in ethanol A 1 g sample disperses and partially dissolves in 100 mL of water at 80° giving a cloudy opalescent solution. (The sample disperses in water more readily if first moistened with alcohol, glycerol, or a saturated solution of glucose or sucrose in water)
Identification of hydrocolloid and predominant type of copolymer	–	Add 4 g of sample to 200 mL of water, and heat the mixture in a water bath at 80°, with constant stirring until dissolved. Replace any water lost by evaporation, and allow the solution to cool to room temperature. The solution becomes viscous and may form a gel. To 50 mL of the solution or gel, add 200 mg of potassium chloride, then reheat, mix well, and cool. A short-textured ('brittle') gel indicates a carrageenan of a predominantly κ-type. A compliant ('elastic') gel indicates a predominantly ι-type.
Infrared absorption	–	Passes test
Purity		
Solvent residues	Not more than 0.1% of methanol, ethanol, propane-2-ol, singly or in combination	Not more than 0.1% of ethanol, isopropanol, or methanol, singly or in combination
Viscosity	Not less than 5 mPa s (1.5% solution at 75°C)	Not less than 5 cp at 75° (1.5% solution)
Loss on drying	Not more than 12% (105°C, 4 h)	Not more than 12% (105° to constant weight)
pH	–	Between 8 and 11 (1 in 100 suspension)
Sulfate	Not less than 15% and not more than 40% on the dried basis (as SO_4)	Not less than 15% and not more than 40% (as SO_4^{2-}) on the dried basis
Ash	Not less than 15% and not more than 40% determined on the dried basis at 550°C	Not less than 15% and not more than 30% on the dried basis
Acid-insoluble ash	Not more than 1% on the dried basis (insoluble in 10% hydrochloric acid)	Not more than 1% Use the ash from the Total ash test
Acid-insoluble matter	Not less than 8% and not more than 15% on the dried basis (insoluble in 1% v/v sulfuric acid)	Not less than 8% and not more than 15% on the dried basis. Use 2 g of sample obtained from part (a) of the procedure for sulfate determination
Low molecular weight carrageenan (Molecular weight fraction below 50 kDa)	Not more than 5%	–
Arsenic	Not more than 3 mg/kg	Not more than 3 mg/kg Determine by the atomic absorption hydride technique. Use Method II for sample preparation
Lead	Not more than 5 mg/kg	Not more than 5 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the method described in Volume 4 (under 'General Methods, Metallic Impurities')

	Commission Regulation (EU) No 231/2012 (E 407a)	JECFA (2007a) (INS 407a)
Mercury	Not more than 1 mg/kg	Not more than 1 mg/kg Determine by the cold vapour atomic absorption technique
Cadmium	Not more than 2 mg/kg	Not more than 2 mg/kg Determine using an AAS/ICP-AES technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the method described in Volume 4 (under 'General Methods, Metallic Impurities')
Microbiological criteria		Initially prepare a 10^{-1} dilution by adding a 50 g sample to 450 mL of Butterfield's phosphate-buffered dilution water and homogenising the mixture in a high speed blender
Total plate count	Not more than 5,000 colonies per gram	Not more than 5,000 CFU/g
Yeast and moulds	Not more than 300 colonies per gram	–
Escherichia coli	Absent in 5 g	Negative in 1 g
Salmonella spp.	Absent in 10 g	Negative per test

Concerning the concentration range of main components of the food additive E 407, the interested party provided information that E 407 produced by alcohol process comprises approximately 90% anhydrous carrageenan, 8% moisture, and 2% inorganic salts (mainly chlorides), while E 407 manufactured by gel press process comprises about 77% anhydrous carrageenan, 8% moisture and up to 15% inorganic salts (mainly chlorides) (Documentation provided to EFSA n. 15). (For details on manufacturing processes see the chapter 3.1.3.1).

However, no such information was provided on the concentration range of main components of the food additive E 407a.

According to the JECFA specifications, 'Articles of commerce may include sugars for standardization purposes, salts to obtain specific gelling or thickening characteristics, or emulsifiers carried over from drum drying processes'. This is also supported with the information from the literature that finished products are made by blending one or more extracts, with or without standardising agents, in order to maintain consistent quality from lot to lot' (FAO, 2003; Blakemore and Harpel, 2010). The Panel noted that this information should be included in the EU specification.

One of the interested parties has provided information in the form of aggregated ranges of concentration and other chemistry data based upon analyses of dozens of samples of the material from member companies for: viscosity (7.0–1,712.0 mPa s), loss on drying (4–12%), sulfates (17.0–36.0%), pH (7.1–11), total ash (19–39%), acid-insoluble ash (0.18–0.89%), acid-insoluble matter (0.34–1.15%), residual alcohol (0.001–0.100%), arsenic (0.005–2.000 mg/kg), lead (0.0001–2.12 mg/kg), mercury (0.0001–1.0000 mg/kg), cadmium (0.005–1.090 mg/kg), total plate count (< 10–5,000 CFU/g), moulds and yeasts (1–300 CFU/g), Coliform bacteria (negative in 5 g) and *Salmonella* (negative in 10 g), (Documentation provided to EFSA n. 15). In addition, the same interested party (Documentation provided to EFSA n. 17) provided raw data on the concentration of toxic elements in 10 non-consecutive batches of carrageenan (E 407) from 5 different manufactures (arsenic: 0.03–1.50 mg/kg, lead: 0.02–< 2.00* mg/kg, cadmium: 0.13–< 2.00* mg/kg and mercury < 0.005*–< 0.05*, and 10 non-consecutive batches of processed Eucheuma seaweed (E 407a) from 6 different manufacturers (arsenic: 0.47–1.70 mg/kg, lead: < 0.04*–< 2.00* mg/kg, cadmium: 0.68–< 2.00* mg/kg and mercury < 0.005*–0.05*. The data provided demonstrated that identity and purity of analysed products complied with the EC specifications (Documentation provided to EFSA n. 15).[20]

In addition, information on range of protein content (N × 6.25%) of 0.06–1.38% was also provided (Documentation provided to EFSA n. 15). According to industry, the proteins in E 407 are denatured during processing from seaweed (high heat + alkali + alcohol). The Panel noted that limits for proteins should be included in the EC specifications (Documentation provided to EFSA n. 15).

[20] * is LOD for specific manufacturer and laboratory used.

According to this interested party (Documentation provided to EFSA n. 15, any enzymatic activity within the live seaweed is either destroyed in the process of the seaweed being dried and/or denatured during manufacturing process, where the seaweed is subjected to high heat and alkaline conditions as well as alcohol precipitation.

Pesticides are not used, nor needed in the production of seaweeds and according to industry (Documentation provided to EFSA n. 15), available analytical data showed that the presence of pesticides was not detectable.

According to Commission Regulation (EU) No 231/2012, formaldehyde may be present as an adventitious impurity up to a maximum of 5 mg/kg. The safety of use of formaldehyde as processing aid during the storage and manufacturing of thickening agents from algae origin was evaluated by EFSA (2007) and it was concluded that exposure to gelling additives containing residual formaldehyde at the levels of 50 mg/kg of additive would be of no safety concern. The Panel noted that maximum limits for formaldehyde (up to a maximum of 5 mg/kg) are established in the current EU Regulation (Reg. EU No 231/2012) for carrageenan (E 407) and processed Eucheuma seaweed (E 407a).

The Panel also noted that according to the information provided to EFSA by industry (Documentation provided to EFSA n. 15 and 17), carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are only produced from dry seaweed and formaldehyde is not used neither for treatment of seaweed nor during the manufacturing process.

Formaldehyde has also been shown to be a natural component of most marine algae including red seaweeds. The levels detected in fresh red seaweeds used for carrageenan extraction were 44 mg/kg for *C. crispus* and 289 mg/kg for *Gigartina stellata* (*Mastocarpus stellatus*) (Yang et al., 1998).

A single laboratory validated colorimetric method that does not use acid hydrolysis (Documentation provided to EFSA n. 48) has been developed for measuring residual formaldehyde in alginate products. According to industry (Documentation provided to EFSA n. 17), attempts to apply this method for measuring formaldehyde in carrageenan (E 407) or processed Eucheuma seaweed (E 407a) were unsuccessful, including validation.

A new high-performance liquid chromatography (HPLC) method for analysis of formaldehyde in carrageenan or processed Eucheuma seaweed has been developed by Hornshøj et al. (2015). Samples are extracted with 2-propanol and reacted with 2,4-dinitrophenylhydrazine (DNPH) to form chromophore formaldehyde-DNPH which is quantified by reversed-phase HPLC with UV detection. The method is single laboratory validated and it has been found to have limit of detection (LOD) of 0.05 mg/kg and limit of quantification (LOQ) of 0.2 mg/kg. Recoveries from spiked samples were 95–107%. Only one sample of E 407 of 20 of E 407 and E 407a tested showed a detectable content of formaldehyde equal to 0.4 $\pm$ 0.06 mg/kg. Based on the results obtained, authors concluded that the formaldehyde content of commercial carrageenan and processed Eucheuma seaweed products are well below the EU maximum limit of 5 mg/kg.

The Panel noted that, according to the data provided, low levels of formaldehyde may be present in the final product in the quantities below the limit of 5 mg/kg set in the EU specifications.

Because of its polysaccharidic nature, carrageenan and processed Eucheuma seaweed can be a substrate for microbiological contamination during storage. The latter has been recently demonstrated by the finding of mycotoxin contaminations of gums (Zhang et al., 2014). The Panel noted that there are only minor differences in the microbiological criteria for carrageenan and processed Eucheuma seaweed between the specifications given by the EU Regulation and those given by JECFA.

The levels of impurities as lead, cadmium, mercury and arsenic, in the four batches analysed, were all below the levels as defined in the Commission Regulation (EU) No 231/2012.

The Panel noted that, according to the EU specifications for carrageenan (E 407) and processed Eucheuma seaweed (E 407a), impurities of the toxic elements arsenic, cadmium, lead and mercury are accepted up to concentrations of 3, 2, 5 and 1, mg/kg, respectively. Contamination at such levels could have a significant impact on the exposure to these metals, for which the exposures already are close to the health-based guidance values or benchmark doses (lower confidence limits) established by the EFSA (EFSA CONTAM Panel, 2009a,b, 2010, 2012a,b,c, 2014).

The Panel also noted that, according to the point (7) of the preamble of the Commission Regulation (EU) No 231/2012: 'Specific purity criteria currently applicable should be adapted by reducing maximum limits for individual heavy metals of interest where feasible and where the JECFA limits are lower than those currently in force. . . . That approach should be departed from for carrageenan (E 407) and processed Eucheuma seaweed (E 407a) (cadmium content) as manufacturers have declared that compliance with stricter Union provisions, reflecting JECFA limits, would not be technically feasible'.

The Panel noted that carrageenan (E 407) and processed Eucheuma seaweed (E 407a) must not contain more than 5% 'low molecular weight carrageenan' with weight-average molecular weight less than 50 kDa. This restriction is not indicated in the JECFA specifications (JECFA, 2014). It is also not clearly stated if the 5% of 'low molecular weight carrageenan' is expressed on the dried basis, as according to the data provided by the industry, E 407 contains 8% moisture (Documentation provided to EFSA n. 15). According to the industry, the Technical Working Group has been unable to develop a validated analytical method in response to this specification during 12 years of research using several methods based on size exclusion chromatography (SEC) and light scattering (LS). None have given reliable and reproducible results. The targets for accuracy and reproducibility were set to achieve a consistent relative standard deviation (RSD) on triplicate sample analysis of not more than 15% and recovery validation within 95–105% of added low molecular weight tail (LMT) of carrageenan (Documentation provided to EFSA n. 15). Six laboratories participated in the preliminary study with the procedure details approved by the Technical Working Group. According to industry, 'Eleven different commercial carrageenan samples, representing different sulphated polygalactose types (nominally κ, λ and ι) made by five different producers, were tested by all laboratories under "Round Robin" conditions. It appears that even under optimum SEC conditions, light scattering detector signal to noise ratio (S/N) in the LMT region is extremely low, and it is this signal upon which molecular weight determination is based' (Documentation provided to EFSA n. 17). According to Spichtig and Austin (2008), 'methods employing light scattering have been unsuccessful in producing reproducible results, probably due to the poor detector response at low masses'.

The molecular weight distributions of 29 samples of food-grade refined carrageenan were studied by the combined GPC/ICP method. According to the authors, no obvious peak of 'poligeenan' was detected, and the LOD was about 5% for 'poligeenan' in the sample of carrageenan (Uno et al., 2001a,b). The Panel noted that the method is not enough sensitive (LOD of 5% degraded carrageenan) and therefore not applicable for quantitative determination of the levels of not more than 5% of low molecular weight carrageenan (molecular weight fraction below 50 kDa) required by the EU specifications.

A single laboratory partially validated method based on high-performance size exclusion chromatography coupled to a refractive index detector (HPSEC-RI) has been employed for the measurement of the low molecular weight fraction of carrageenan in food-grade carrageenan ingredients and in a carrageenan-containing finished product (a jelly). Their use of carrageenan standards makes the application of light scattering (LS) unnecessary, thus removing the signal to noise ratio problem. However, as indicated by the authors, degraded carrageenan standards are difficult to make and may spoil on storage. LOD of the method was not reported. Over the course of half a year, 19 measurements were made on a reference carrageenan that demonstrated the method had good reproducibility (coefficient of variation (CV) for the low molecular weight fraction was 2.9%) in a single laboratory. Accuracy was difficult to assess due to the poor sensitivity of the two reference methods (HPSEC-RI-MALS systems) below 50 kD. According to the authors, a robust solution would be to have available a supply of well characterised carrageenan standards for HPSEC-RI calibration. An interlaboratory study, such as the one carried out by one of the interested parties, would be required to ascertain that the system also gives reproducible results in other laboratories. Applied to a number of different food-grade carrageenan samples, it was found that, in general, the low molecular weight fraction represents less than 8% of the total carrageenan and that around half of the tested samples was within the specified 5% limit (Spichtig and Austin, 2008; Documentation provided to EFSA n. 18). The Panel noted that, according to the data presented, half of the samples of carrageenan tested by Spichtig and Austin (2008) did not comply with the EU specification for low molecular weight carrageenan.

Investigation of ultrafiltration membranes in an attempt to separate and quantify the LMT carrageenan concluded that current commercially available membranes are not porous enough for the size and shape of LMT molecules (Documentation provided to EFSA n. 17).

According to information from one of the interested parties (Documentation provided to EFSA n. 19), 'in native carrageenan, this LMT region represents less than 10% of the total carrageenan molecule. Although there is a Commission specification of not more than 5% at less than 50 kDa, in practice there is no validated analytical method that can accurately quantify the percent of LMT present'.

The Panel thus noticed that, according to the interested parties, an interlaboratory validated analytical method to accurately measure the low molecular weight distributions of carrageenan was not fully developed or available at present.

Based on the data provided following general and specific calls for data (Documentation provided to EFSA n. 17 and 19), the overlapping between the low weight-average molecular weight tail of carrageenan and high weight-average molecular weight tail of poligeenan is expected to increase with

the decrease of the weight-average molecular weight of the carrageenan preparation. For example, for the carrageenan with the weight-average molecular weight of around 770 kDa (Figure 2), the overlap would encompass from approximately 30 kDa up to approximately 200 KDa molecules. Although for a commercially available carrageenan with a weight-average molecular weight of 200 kDa, as referred to by an interested party, the preparation would encompass a larger fraction with a molecular weight below 50 kDa.

Based on the information provided on weight-average molecular weights of commercially available carrageenan preparations, the Panel concluded that it cannot be excluded that carrageenan preparations encompassing considerable amounts of low molecular weight molecules could be used as food additive.

Therefore, with respect to the definition of carrageenan E 407 and Eucheuma seaweed (E 407a) in the Commission Regulation (EU) No 231/2012, the weight-average molecular weight range should be specified in a narrow way avoiding a significant overlap with the molecular weight range of poligeenan.

With respect to the purity criteria for carrageenan E 407 and processed Eucheuma seaweed (E 407a) in the Commission Regulation (EU) No 231/2012, the Panel noted the urgent need to develop a validated method to quantify the low molecular weight carrageenan with the defined limit of 5%.

3.1.3. Manufacturing process

3.1.3.1. Carrageenan (E 407)

According to Commission Regulation (EU) No. 231/1012, carrageenan is obtained from algae of the families Gigartinaceae, Solieriaceae, Hypneaceae and Furcellariaceae. Generally, the industrial sources of carrageenan are various species of red *algae* of the class Rhodophyceae, mainly *E. cottonii, E. spinosum, C. crispus, Chondrus ocellatus, Hypnea musciformis* and several *Gigartina, Iridaea* and *Furcellaria* species (Imeson, 2000; van de Velde and De Ruiter, 2002; Marburger, 2003). The algae are harvested either in nature or produced by seaweed farming (van de Velde and De Ruiter, 2002).

According to the information provided by an interested party (Documentation provided to EFSA n. 15 and 17), carrageenan (E 407) is only produced from dry seaweed and formaldehyde is not used in the production of carrageenan (E 407), neither for treatment of seaweed nor during the manufacturing process.

After the seaweeds are identified and selected to make particular extract they are washed to remove sand, salts and other foreign materials. It is then heated with water containing an alkali, for several hours in order to increase gel strength in the final product. Non-dissolved seaweed is removed by centrifugation or by a coarse filtration. The solution is then filtered again, in a pressure filter and at this stage, the solution contains 1–2% carrageenan and it is usually concentrated to 2–3% by vacuum distillation and ultrafiltration. There are two methods ('alcohol-precipitation method' and 'gel method') for recovering a solid. 'An alcohol-precipitation method' can be used for any of the carrageenan, while 'a gel method' can be used exclusively for κ-carrageenan. In the alcohol-precipitation method, isopropanol is added until all the carrageenan is precipitated as a fibrous coagulum that is then separated by centrifugation or by sieving through a fine sieve. The coagulum is pressed to remove solvent, washed with alcohol and then dried and milled to an appropriate particle size (80 mesh or finer). The 'gel method' relies on the ability of κ-carrageenan to form a gel with potassium salts. The gel may be formed in various ways. For the freeze-thaw process, it is convenient to form it as spaghetti-like pieces by passing the carrageenan solution through fine holes into a potassium chloride solution. The fine 'spaghetti' is collected and washed with potassium chloride, pressed to remove excess liquid and then frozen. When allowed to thaw, separation of water occurs by syneresis, the pieces are washed with potassium chloride, chopped up and dried in a hot air dryer. Inevitably the product contains some potassium chloride. The alternative to freeze-thaw is to squeeze out water from the gel by applying pressure to it. After squeezing for several hours the sheets of gel are chopped, dried in a hot air dryer and milled to an appropriate particle size. Finished products are made by blending one or more extracts, with or without standardising agents, in order to maintain consistent quality from lot to lot (FAO, 2003; Blakemore and Harpel, 2010).

According to the information provided by one of the interested parties (Documentation provided to EFSA n. 17), calcium hypochlorite is not used as a bleaching agent in the manufacturing process of the food additive carrageenan (E 407). Provided results of analysis of the content of semicarbazide in 10 commercial batches of carrageenan from 5 manufacturers were in the range of < 1–2 μg/kg. At maximum use in food of 2%, maximum semicarbazide contribution by carrageenan with 2 μg/kg semicarbazide to any food product would be 0.08 μg/kg (Documentation provided to EFSA n. 17).

The following flow chart (Blakemore and Harpel, 2010) (figure 4) details the manufacturing process for E 407.

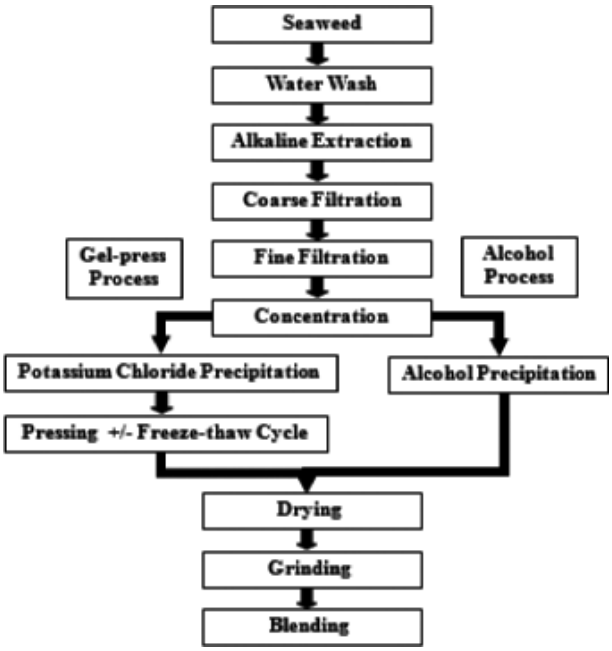


Figure 4: Carrageenan manufacturing process flow chart (re-produced from Blakemore and Harpel, 2010). (Documentation provided to EFSA n. 15)

3.1.3.2. Processed Eucheuma seaweed (E 407a)

According to Commission Regulation (EU) No 231/2012, the industrial sources of processed Eucheuma seaweed are strains of the seaweeds *E. cottonii* and *E. spinosum*, of the class Rhodophyceae.

According to the information provided by one of the interested parties (Documentation provided to EFSA n. 15 and 17), processed Eucheuma seaweed (E 407a) are only produced from dry seaweed and formaldehyde is not used in the production of processed Eucheuma seaweed (E 407a), neither for treatment of seaweed nor during the manufacturing process.

For the manufacturing process of processed Eucheuma seaweed (E 407a), the seaweed is treated with a potassium hydroxide solution for a short time at 70–80°C. This temperature is not high enough to allow the carrageenan to be extracted, but most of the low-molecular-mass material is removed. According to some information found in the literature, 'the treated material is then thoroughly washed with water to remove residual salts and further washed with alcohol, dried and milled to a powder. The alcohols that may be used during purification are restricted to methanol, ethanol and isopropanol' (JECFA, 2001; van de Velde and De Ruiter, 2002).

The manufacture processes of processed Eucheuma seaweed (E 407a) and carrageenan (E 407) are similar. While during carrageenan production the carrageenan is solubilised to remove solids, the processed Eucheuma seaweed process leaves carrageenan within the seaweeds cellulosic structural matrix. Therefore, processed Eucheuma seaweed contains algal cellulose, in contrast to carrageenan. All further purification steps are comparable (Blakemore and Harpel, 2010).

According to EFSA AFC Panel (EFSA, 2005), it is stated that processed Eucheuma seaweed (E 407a) is sometimes bleached with calcium hypochlorite resulting in higher concentrations of the reaction by-product semicarbazide; industry initiated trials to replace calcium hypochlorite with an alternative bleaching agent for the production of processed Eucheuma seaweed (E 407a).

According to information received by industry (Documentation provided to EFSA n. 17), three manufacturers of processed Eucheuma seaweed (E 407a) were identified as currently using calcium hypochlorite for bleaching and one manufacturer uses hydrogen peroxide for bleaching. Commercial samples from these manufacturers were analysed for semicarbazide for comparison, along with unbleached batches. The results are shown in Table 5. At maximum use in food of 2%, maximum semicarbazide contribution by processed Eucheuma seaweed (E 407a) with 39 μg/kg semicarbazide to any food product would be 0.78 μg/kg (Documentation provided to EFSA n. 17).

Table 5: Semicarbazide levels in processed Eucheuma seaweed (E 407a) after different bleaching treatments from different manufactures

Manufacture	Treatment	Semicarbazide (μg/kg)
–	(all lots washed after treatment)	
F	No calcium hypochlorite/peroxide	8
G	No calcium hypochlorite/peroxide	4
H	No calcium hypochlorite/peroxide	12
I	No calcium hypochlorite/peroxide	11
J	No calcium hypochlorite/peroxide	5
G	800 mg/L calcium hypochlorite	37
G	1,000 mg/L calcium hypochlorite	20
J	1,000 mg/L calcium hypochlorite	39
H	5,000 mg/L peroxide	17
H	2,500 mg/L peroxide	22

For processed Eucheuma seaweed prepared using a bleaching step, for the 25 batches reported by industry to EFSA AFC Panel (2005), the range for semicarbazide was 9–380 μg/kg with a mean of 65 μg/kg.

According to the EFSA AFC Panel (2005), 'for the food additive carrageenan, that may become contaminated with semicarbazide at a mean concentration of 65 μg/kg from use of hypochlorite in the production process, if consumption was up to the Acceptable Daily Intake (ADI) for carrageenan of 75 mg/kg bw per day then the intake of semicarbazide from this source could be up to 0.005 μg/kg bw per day'. Based on the overall weight of evidence, the AFC Panel concluded that 'the weak genotoxicity exerted by semicarbazide *in vitro* is not expressed *in vivo*. A large margin of at least 5 orders of magnitude exists between the dose causing tumours in experimental animals and human exposure, including that of infants'. Therefore, the AFC Panel concluded that 'the issue of carcinogenicity is not of concern for human health at the concentrations of semicarbazide encountered in food'.

The Panel noted that the carcinogenic effects of semicarbazide referred to by the AFC Panel were observed in a sensitive mouse strain but not in rat (Davies et al., 2000), and that semicarbazide was classified by IARC in Group 3 based on 'limited evidence' of carcinogenicity in experimental animals and 'no data' in humans (IARC, 1987). The Panel further noted that the currently reported levels on semicarbazide are below levels reported to the AFC Panel and thus would not be of toxicological concern referring to the existing ADI. The Panel also noted results from other studies (Maranghi et al., 2009, 2010) investigating effects in juvenile animals and on endocrine disruption, which did not allow the derivation of a NOAEL without further investigations.

The following flow chart (Blakemore and Harpel, 2010) (Figure 5) details the manufacturing process for processed Eucheuma seaweed (E 407a).

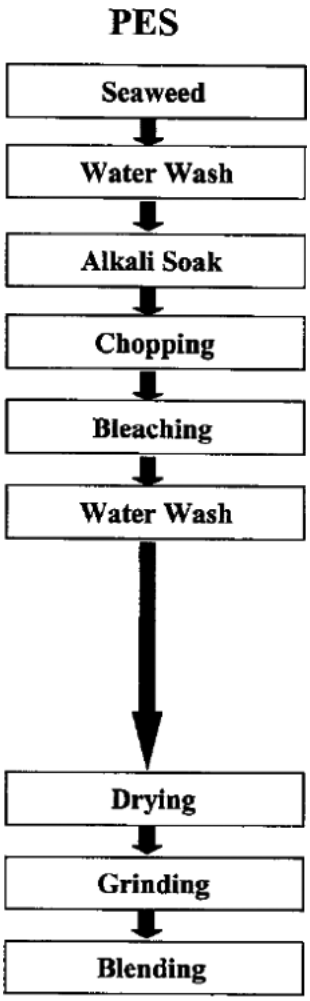


Figure 5: Processed Eucheuma seaweed manufacturing process flow chart (re-produced from Blakemore and Harpel, 2010; Documentation provided to EFSA n. 15)

3.1.4. Methods of analysis in food

3.1.4.1. Carrageenan (E 407)

A single-laboratory validated method to analyse carrageenan mixed into the diet for animals of an experimental study was verified (Documentation provided to EFSA n. 37). Carrageenan concentration in powdered rodent diet was determined, after extraction with water at approximately 75°C and shaking in water bath for 20 min. After centrifugation, the viscosity of supernatant was measured (no information on the method used). A linear calibration line was obtained, and the limit of detection (LOD) for the carrageenan was determined to be 0.42 g/100 g diet.

There is a number of analytical methods available for determination of carrageenan in foodstuff, including: gas chromatography (GC), HPLC, colorimetry, electrophoresis, immunoassays, and potentiometry. An overview of these methods was presented in a review by Roberts and Quemener (1999).

Gas chromatography

An analytical method for carrageenan and further polysaccharides (locust bean gum, guar gum, gum arabic, tragacanth, arabinogalactan, agar, furcellaran, xanthan) in dairy products was described by Glueck and Thier (1980). Thereby polysaccharides are extracted from foodstuff, and then fat, starch, milk proteins and carbohydrates are removed by extraction or degradation. The resulting polysaccharide fraction is analysed by GC after hydrolysis with trifluoroacetic acid, derivatisation of the resulting monosaccharides with hydroxylamine hydrochloride and acetic acid anhydride to form the aldonitrilacetate derivatives. The polysaccharides can be qualitatively identified by their characteristic monosaccharide pattern, and quantified via the single monosaccharide peaks. In the case of

carrageenan, the only hydrolysis product is galactose. For the polysaccharides, recoveries of 80–90% were obtained when adding 0.05% of the thickeners to skim milk or 1–2% to mixtures of ice cream or pudding constituents (Glueck and Thier, 1980). The analytical method without extraction from foodstuff was previously described by Mergenthaler and Scherz (1976).

In a later investigation the analytical procedure described by Glueck and Thier (1980) was improved by Preuss and Thier (1982, 1983). Changes in the separation of interfering substances (fats, proteins and starch) allowed the quantitative determination of carrageenan in a variety of foods like blancmanger powder, glaze, fruit ice, and cream cheese. Recoveries for most of the thickeners and gums are about 60–85% with a CV of 2–8 %. The Panel noted that both methods described do not allow to distinguish between agar and carrageenan as both polysaccharides consist only of galactose units.

Mergenthaler and Scherz (1980) developed an analytical method which allows to distinguish carrageenan from agar (and from other polysaccharides). After extraction of fat and proteins, the polysaccharides are eluted through a chromatographic column filled with diethylaminoethyl (DEAE)-cellulose, where carrageenan is separated from other polysaccharides inclusive agar. The resulting fractions can be further analysed either by thin layer chromatography or, after derivatisation, by GC.

The Panel noted that the above mentioned methods have been published without indication of the LOD.

Size exclusion chromatography methods

Hunziker and Miserez (1980) published a method for quantitative determination of different polysaccharides using gel permeation chromatography. After extraction of fat, carrageenan is purified by precipitation with methanol, and the precipitate is further washed and dried. The polysaccharide is chromatographed as 0.04 % solution.

A method based on HPSEC-RI has been employed for the measurement of the low molecular weight fraction of carrageenan in food-grade carrageenan ingredients and in a carrageenan-containing finished product (a jelly) (Spichtig and Austin, 2008). According to the authors, 'over the course of half a year, 19 measurements were made on a reference carrageenan and the results demonstrated that the method had good reproducibility (CV for the low molecular weight fraction was 2.9%) in a single laboratory. Applied to a number of different carrageenan ingredients, it was found that, in general, the low molecular weight fraction represents less than 8% of the total carrageenan in ingredients, and under the correct conditions increases little during food processing'.

Electrophoretic methods

An electrophoretic method for qualitative and quantitative analysis of gelling agents in food (pudding, milk-based baby food, sugar fruits, ice cream, ketchup, cream stabiliser) was published by Pechanek et al. (1982). After removal of fat, starch and proteins, carrageenan is precipitated and separated via electrophoresis using cellulose acetate membrane and stained with toluidine blue. The polysaccharides are quantified using a scanner. Lowest reported LOD for carrageenan was 40 ng.

Colorimetric methods

Soedjak (1994) analysed carrageenan in milk as methylene blue complex which can be determined photometrically. The interaction between methylene blue and polyanions is reversible, electrostatic, and stoichiometric (1:1 ratio between the anionic sites and the bound dye molecules). The method was found to be sensitive, capable of determination between 0.2 and $2.0 \cdot 10^{-3}$% polyanion of the sample. Due to the high sensitivity of the method, potentially interfering compounds (e.g. acids, sugars, salts, milk, proteins, dyes, emulsifiers, and neutral hydrocolloids) may be diluted out.

A similar method using alcian blue as complexation agent was published by Yabe et al. (1991). Carrageenan-alcian blue complex is precipitated, the precipitate dissolved in monoethanolamine and determined colorimetrically at 615 nm. Recoveries from jellies and salad dressings were > 90%, the detection limit was 0.05%.

Potentiometric methods

Carrageenan was determined at the concentration levels of 0.2–1.5% (m/m) in a variety of food products (e.g. cream, chocolate, caramel, ice cream, and salad dressing) using potentiometric titration of carrageenan with protamine titrant. For monitoring potentiometric titration polymeric matrix tubular membrane sensors were used. Reported limit of detection (LOD) was 0.7 μg/mL (Hassan et al., 2002).

Immunoassay methods

Haines and Patel (2005) developed an enzyme-linked immunosorbent assay (ELISA) for quantification of carrageenan in different foods (water jelly, milk jelly, salad dressing, chocolate milk, and meat pate). After extraction of fats and digesting proteins with protease, carrageenan is bound on a solid phase column containing sheep immunoglobulin G (IgG) antibody to carrageenan, and then eluted for measurement. No cross reaction with competing polysaccharides was observed. The method was inter-laboratory validated in accordance with an International AOAC PVM-approved procedure (AOAC, 1998). The LOD was 0.5 μg/mL and the mean recovery was in the range 69–102%. The results were highly repeatable (< 10% CV) and reproducible (< 15% CV). The Panel noted that it was not reported whether the anti-carrageenan antibody also recognised poligeenan or other 'degraded' carrageenan.

The Panel noted that acceptable methods were available for the determination of carrageenan in foodstuff.

3.1.4.2. Processed Eucheuma seaweed (E 407a)

There were no validated methods available which are specific for the analysis of processed Eucheuma seaweed (E 407a) in foodstuff and discriminate between processed Eucheuma seaweed and carrageenan.

However, an analysis method of processed Eucheuma seaweed (E 407a) mixed into the diet for animals of an experimental study was verified by (Documentation provided to EFSA n. 37). For details, see Section 3.1.4.1.

In addition, a number of other analytical methods were developed for the determination of carrageenan, the main component of processed Eucheuma seaweed (E 407a). These methods include GC, HPLC, colorimetry, electrophoresis, immunoassays and potentiometry. An overview is presented in the review of Roberts and Quemener (1999). See Section 3.1.4.1.

3.1.5. Stability of the substance, and reaction and fate in food

According to JECFA (2015), 'the stability of carrageenan in foods is influenced by several factors, such as pH, direct structural bridging between the negatively charged carrageenan and positively charged protein sites, and indirect structural bridging with negatively charged protein sites via divalent cations such as calcium, through hydrogen bonding and through carrageenan–carrageenan helical interactions' (JECFA, 2015).

According to Weiner (2014), a direct polyanion carrageenan and polycation protein interaction can occur below the isoelectric point of the protein, resulting in gelation or precipitation. Also, this direct carrageenan–protein bonding occurs above the isoelectric point, but to a lesser degree as the pH is increased. Above the isoelectric point, positively charged cations, such as calcium or other polyvalent cations, form a bridge across the negatively charged sulfate groups on carrageenan to the negatively charged carboxyl groups on the protein, forming stable gels. In addition, carrageenan–carrageenan molecules interact to form helical bridges, adding strength to the gels. In the absence of protein, carrageenan forms a loose random conformation in water solution rather than the structured helical coil conformation formed in the presence of protein (Blakemore and Harpel, 2010).

According to Spichtig and Austin (2008), pH seems to be a critical factor during food processing with respect to possible formation of low molecular weight carrageenan and pH levels less than 4.0 should be avoided.

According to industry, 'the presence of other food components can have significant positive impacts on carrageenan stability, particularly those that interact (cross-link) with the carrageenan (e.g. proteins, locust bean gum) or tighten the carrageenan molecular structure (e.g. salts, high solids). These food conditions therefore provide carrageenan added stability and protection from degradation' (Documentation provided to EFSA n. 17;18).

Carrageenan is sensitive to acid and oxidative breakdown. Acid-catalysed hydrolysis occurs at the glycosidic linkage, leading to depolymerisation (van de Velde and De Ruiter, 2002). According to industry, at pH values above 6, carrageenan is very stable even at higher temperatures. However, no detailed information was provided. Isolated carrageenan powders are slightly alkaline (pH 8–10), they are highly stable during storage. The industry reported that, 'when carrageenan is stored under cool dry conditions it is stable in excess of two years' (Marinalg, 2006).

κ-Carrageenan is the most sensitive type of carrageenan to exposure at low pH. The rate of hydrolysis (as determined by specific viscosity measurements) of κ-carrageenan is dependent on

carrageenan state. The use of buffers (e.g. citrate) is recommended for lower pH applications, and hydrolysis rates will show minor variations depending on the buffer system and concentration used (Documentation provided to EFSA n. 17).

Rasmussen (1974) confirmed that heat treatment at pH 7.0 of carrageenan at 100 and 121°C did not affect gel functionality. Processing at 85°C for 20 min at pH 3.5 gave a 50% decrease in gel strength for κ-carrageenan but no decrease in gel strength for ι-carrageenan.

A hydrolytic effect on carrageenan was demonstrated in a synthetic milk salt system with an initial pH of 6.70. The average molecular weight decreased by 42% after 20 min of heat treatment at 122°C (Badui, 1978). When heating carrageenan in sodium cacodylate buffer (pH 7) for 60 min at 122°C, the average molecular weight decreased by 17.3% (Desai and Hansen, 1986).

There were no studies on the stability of processed Eucheuma seaweed (E 407a) available. However, the main components of processed Eucheuma seaweed (E 407a) are κ- and ι- carrageenan that are subject to the same stability considerations. The cellulose in processed Eucheuma seaweed (E 407a) is also very stable in the pH range of processed foods (Marinalg, 2006).

The Panel noted that degradation of κ-carrageenan under mild acid conditions (0.1 M sulfuric acid at 60°C for 1.5 h) has been described by Yang et al. (2009).

The Panel further noted that only limited information on the stability of carrageenan in food was available. No adequate data on stability of carrageenan and/or processed Eucheuma seaweed addressing the usual variation of parameters (temperature, pH) relevant for the authorised food uses were available. Information on possible degradation products under acidic conditions in relevant food products was missing.

3.2. Authorised uses and use levels

Maximum levels of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) have been defined in Annex II to Regulation (EC) No 1333/2008[2] on food additives, as amended. These levels are defined by the Panel as 'maximum permitted levels (MPLs)' in this document.

Currently, carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised food additives in the EU at *quantum satis* (QS) in almost all foods. Specifically, carrageenan (E 407) is authorised in jam, jellies and marmalades and sweetened chestnut puree (04.2.5.2), other similar fruit or vegetable spreads (04.2.5.3) at MPL 10,000 mg/Kg and follow-on formulae (13.1.2), other foods for young children (13.1.4), dietary foods for infants for special medical purposes and special formulae for infants (13.1.5.1) and dietary foods for babies and young children for special medical purposes (13.1.5.2) at MPL 300 mg/Kg. Carrageenan E 407 and processed Eucheuma seaweed (E 407a) are included in the Group I of food additives authorised at (QS).

Table 6 summarises foods that are permitted to contain carrageenan (E 407) and processed Eucheuma seaweed (E 407a) and the corresponding MPLs as set by Annex II to Regulation (EC) No 1333/2008.

Table 6: MPLs of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) in foods according to the Annex II to Regulation (EC) No 1333/2008

FCS category number	Food category names	E-number/ group	Restrictions/ exception	MPL (mg/L or mg/kg as appropriate)
01.3	Unflavoured fermented milk products, heat-treated after fermentation	Group I		Quantum satis
01.4	Flavoured fermented milk products including heat-treated products	Group I		*Quantum satis*
01.5	Dehydrated milk as defined by Directive 2001/114/EC	E 407		*Quantum satis*
01.6.1	Unflavoured pasteurised cream (excluding reduced fat creams)	E 407		*Quantum satis*
01.6.2	Unflavoured live fermented cream products and substitute products with a fat content of less than 20%	E 407		*Quantum satis*

FCS category number	Food category names	E-number/ group	Restrictions/ exception	MPL (mg/L or mg/kg as appropriate)
01.6.3	Other creams	Group I		*Quantum satis*
01.7.1	Unripened cheese excluding products falling in category 16	Group I	Except mozzarella	*Quantum satis*
01.7.5	Processed cheese	Group I		*Quantum satis*
01.7.6	Cheese products (excluding products falling in category 16)	Group I		*Quantum satis*
01.8	Dairy analogues, including beverage whiteners	Group I		*Quantum satis*
02.2.2	Other fat and oil emulsions including spreads as defined by Council Regulation (EC) No 1234/2007 and liquid emulsions	Group I		*Quantum satis*
02.3	Vegetable oil pan spray	Group I		*Quantum satis*
03	Edible ices	Group I		*Quantum satis*
04.2.1	Dried fruits and vegetables	Group I		*Quantum satis*
04.2.2	Fruit and vegetables in vinegar, oil, or brine	Group I		*Quantum satis*
04.2.4.1	Fruit and vegetable preparations excluding compote	Group I		*Quantum satis*
04.2.5.2	Jam, jellies and marmalades and sweetened chestnut puree as defined by Directive 2001/113/EC	E 407	Maximum individually or in combination with E 400–404, E 406, E 407, E 410, E 412, E 415 and E 418	*10,000 mg/kg*
04.2.5.3	Other similar fruit or vegetable spreads	E 407	Maximum individually or in combination with E 400–404, E 406, E 407, E 410, E 412, E 415 and E 418	*10,000 mg/kg*
04.2.5.4	Nut butters and nut spreads	Group I		*Quantum satis*
04.2.6	Processed potato products	Group I		*Quantum satis*
05.1	Cocoa and Chocolate products as covered by Directive 2000/36/EC	Group I	Only energy-reduced or with no added sugars	*Quantum satis*
05.2	Other confectionery including breath refreshening microsweets	Group I	E 407 and E 407a may not be used in jelly mini-cups, defined, for the purpose of this Regulation, as jelly confectionery of a firm consistence, contained in semi rigid mini-cups or mini-capsules, intended to be ingested in a single bite by exerting pressure on the mini-cups or mini-capsule to project the confectionery into the mouth	*Quantum satis*
05.3	Chewing gum	Group I		*Quantum satis*
05.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	Group I		*Quantum satis*
06.2.2	Starches	Group I		*Quantum satis*

FCS category number	Food category names	E-number/ group	Restrictions/ exception	MPL (mg/L or mg/kg as appropriate)
06.3	Breakfast cereals	Group I		*Quantum satis*
06.4.2	Dry pasta	Group I	Only gluten free and/ or pasta intended for hypoproteic diets in accordance with Directive 2009/39/EC	*Quantum satis*
06.4.4	Potato gnocchi	Group I	Except fresh refrigerated potato gnocchi	*Quantum satis*
06.4.5	Fillings of stuffed pasta (ravioli and similar)	Group I		*Quantum satis*
06.5	Noodles	Group I		*Quantum satis*
06.6	Batters	Group I		*Quantum satis*
06.7	Pre-cooked or processed cereals	Group I		*Quantum satis*
07.1	Bread and rolls	Group I	Except products in 7.1.1 and 7.1.2	*Quantum satis*
07.2	Fine bakery wares	Group I		*Quantum satis*
08.2	Meat preparations as defined by Regulation (EC) No 853/2004	E 407/ E 407a	Only preparations in which ingredients have been injected; meat preparations composed of meat parts that have been handled differently: minced, sliced or processed and that are combined together. Except bifteki, soutzoukaki, kebap gyros and souvlaki	*Quantum satis*
08.3.1	Non-heat-treated processed meat	Group I		*Quantum satis*
08.3.2	Heat-treated processed meat	Group I	Except foie gras, foie gras entier, blocs de foie gras, Libamáj, libamáj egészben, libamáj tömbben	*Quantum satis*
08.3.3	Casings and coatings and decorations for meat	Group I		*Quantum satis*
09.2	Processed fish and fisheries products including molluscs and crustaceans	Group I		*Quantum satis*
09.3	Fish roe	Group I	Only processed fish roe	*Quantum satis*
10.2	Processed eggs and egg products	Group I		*Quantum satis*
11.2	Other sugars and syrups	Group I		*Quantum satis*
11.4.1	Table-top Sweeteners in liquid form	E 407		*Quantum satis*
11.4.2	Table-top Sweeteners in powder form	E 407		*Quantum satis*
12.1.2	Salt substitutes	Group I		*Quantum satis*
12.2.2	Seasonings and condiments	Group I		*Quantum satis*
12.3	Vinegars and diluted acetic acid (diluted with water to 4–30% by volume)	Group I		*Quantum satis*
12.4	Mustard	Group I		*Quantum satis*
12.5	Soups and broths	Group I		*Quantum satis*
12.6	Sauces	Group I		*Quantum satis*

FCS category number	Food category names	E-number/ group	Restrictions/ exception	MPL (mg/L or mg/kg as appropriate)
12.7	Salads and savoury-based sandwich spreads	Group I		*Quantum satis*
12.8	Yeast and yeast products	Group I		*Quantum satis*
12.9	Protein products, excluding products covered in category 1.8	Group I		*Quantum satis*
13.1.2	Follow-on formulae as defined by Directive 2006/141/EC	E 407	If more than one of the substances E 407, E 410 and E 412 is added to a foodstuff, the maximum level established for that foodstuff for each of those substances is lowered with that relative part as is present of the other substances together in that foodstuff	*300 mg/kg*
13.1.4	Other foods for young children	E 407	If more than one of the substances E 407, E 410 and E 412 is added to a foodstuff, the maximum level established for that foodstuff for each of those substances is lowered with that relative part as is present of the other substances together in that foodstuff	*300 mg/kg*
13.1.5.1	Dietary foods for infants for special medical purposes and special formulae for infants	E 407	If more than one of the substances E 407, E 410 and E 412 is added to a foodstuff, the maximum level established for that foodstuff for each of those substances is lowered with that relative part as is present of the other substances together in that foodstuff	*300 mg/kg*
13.1.5.2	Dietary foods for babies and young children for special medical purposes as defined in Directive 1999/21/EC	E 407	If more than one of the substances E 407, E 410 and E 412 is added to a foodstuff, the maximum level established for that foodstuff for each of those substances is lowered with that relative part as is present of the other substances together in that foodstuff	*300 mg/kg*

FCS category number	Food category names	E-number/ group	Restrictions/ exception	MPL (mg/L or mg/kg as appropriate)
13.2	Dietary foods for special medical purposes defined in Directive 1999/21/ EC (excluding products from food category 13.1.5)	Group I		*Quantum satis*
13.3	Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)	Group I		*Quantum satis*
13.4	Foods suitable for people intolerant to gluten as defined by Regulation (EC) No 41/2009	Group I	Including dry pasta	*Quantum satis*
14.1.2	Fruit juices as defined by Directive 2001/ 112/EC and vegetable juices	Group I	Only vegetable juices	*Quantum satis*
14.1.3	Fruit nectars as defined by Directive 2001/112/EC and vegetable nectars and similar products	Group I	Only vegetable nectars	*Quantum satis*
14.1.4	Flavoured drinks	Group I		*Quantum satis*
14.1.5.2	Other	Group I	Excluding unflavoured leaf tea; including flavoured instant coffee	*Quantum satis*
14.2.3	Cider and perry	Group I		*Quantum satis*
14.2.4	Fruit wine and made wine	Group I		*Quantum satis*
14.2.5	Mead	Group I		*Quantum satis*
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008	Group I	Except whisky or whiskey	*Quantum satis*
14.2.7.1.	Aromatised wines	Group I		*Quantum satis*
14.2.7.2.	Aromatised wine-based drinks	Group I		*Quantum satis*
14.2.7.3.	Aromatised wine-product cocktails	Group I		*Quantum satis*
14.2.8	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15% of alcohol	Group I		*Quantum satis*
15.1	Potato-, cereal-, flour- or starch-based snacks	Group I		*Quantum satis*
15.2	Processed nuts	Group I		*Quantum satis*
16	Desserts excluding products covered in category 1, 3 and 4	Group I		*Quantum satis*
17.1[(a)]	Food supplements supplied in a solid form including capsules and tablets and similar forms, excluding chewable forms	Group I		*Quantum satis*
17.2[(a)]	Food supplements supplied in a liquid form	Group I		*Quantum satis*
17.3[(a)]	Food supplements supplied in a syrup-type or chewable form	Group I		*Quantum satis*
18	Processed foods not covered by categories 1 to 17, excluding foods for infants and young children	Group I		*Quantum satis*

MPL: maximum permitted level.

(a): FCS 17 refers to food supplements as defined in Directive 2002/46/EC of the European Parliament and of the Council excluding food supplements for infants and young children.

According to Annex II of Regulation (EC) No 1333/2008, carrageenan (E 407) and processed Eucheuma seaweed (E 407a) may be standardised with sugars, on condition that this is stated in addition to the number and designation.

According to Annex III, Part 1 of Regulation (EC) No 1333/2008, carrageenan (E 407) is authorised as carrier in all food additives at the level of QS.

According to Annex III, Part 3, carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised in enzyme preparations at the level of QS.

In addition, according to Annex III Part 5 of Regulation (EC) No 1333/2008, carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised in nutrients except nutrients intended to be used in foodstuffs for infants and young children at the level of QS.

3.3. Exposure data

3.3.1. Reported use levels or data on analytical levels of carrageenan (E 407) and processed Eucheuma seaweed (E 407a)

Most food additives in the EU are authorised at a specific MPL. However, a food additive may be used at a lower level than the MPL. Therefore, information on actual use levels is required for performing a more realistic exposure assessment, especially for those food additives for which no MPL is set and which are authorised according to QS.

In the framework of Regulation (EC) No 1333/2008 on food additives and of Commission Regulation (EU) No 257/2010 regarding the re-evaluation of approved food additives, EFSA issued a public call[21] for food additive usage level and/or concentration data in food and beverages intended for human consumption, including old call for data[22] on carrageenan (E 407) and Eucheuma seaweed (E 407a). In response to these calls, updated information on the actual use levels of carrageenan (E 407) and Eucheuma seaweed (E 407a) in foods was made available to EFSA by industry. Only few analytical data on the concentration of carrageenan (E 407) in foods were made available by Member States.

Summarised data on reported use levels in foods provided by industry

Industry provided EFSA with data on use levels (n = 457) of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) (n = 105) in foods for 50 out of the 79 food categories in which E 407 and 28 out of the 71 food categories in which E 407a are, respectively, authorised.

Updated information on the actual use levels of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) in foods was made available to EFSA by the Association of the European Self-Medication Industry (AESGP) (Documentation provided to EFSA n. 20), Associazione Industriali delle Carni e dei Salumi (Documentation provided to EFSA n. 21), BABBI Confectionary Industry (Documentation provided to EFSA n. 22), Delixia s.r.l. (Documentation provided to EFSA n. 23, Interested party 1 (Documentation provided to EFSA n. 28), EUROGUM A/S (Documentation provided to EFSA n. 24), Fabricante Embutidos del centro SA (España) (Documentation provided to EFSA n. 25), FoodDrinkEurope (FDE) (Documentation provided to EFSA n. 26), the International Chewing Gum Association (ICGA) (Documentation provided to EFSA n. 27), Intertek Scientific & Regulatory Consultancy (Documentation provided to EFSA n. 29), Rudolf Wild GmbH & Co (Documentation provided to EFSA n. 30), and Specialised Nutrition Europe (SNE) (Documentation provided to EFSA n. 31).

The Panel noted that some data providers (Documentation provided to EFSA n. 24) are not a food industry using carrageenan and processed Eucheuma seaweed in their food products but food additive producers. Usage levels reported by food additive producers are not considered at the same level as those provided by food industry. Food additive producers might recommend usage levels to the food industry but the final levels might, ultimately, be different. Therefore, unless food additive producers confirm that these levels are used by food industry, they are not considered in the refined exposure scenario. Data from food additive producers will only be used in the *maximum level exposure assessment scenario* in case of QS authorisation when no data are available from food industry. In this way, the most complete exposure estimates are calculated.

For instance, for one of the data provider (Documentation provided to EFSA n. 24), all the submitted data are theoretical amounts suggested or recommended; they are 'based on their own

[21] Call for food additives usage level and/or concentration data in food and beverages intended for human consumption. Available online: https://www.efsa.europa.eu/en/dataclosed/call/datex140310

[22] Call for scientific data on food additives permitted in the EU and belonging to the functional classes of emulsifiers, stabilisers and gelling agents Published: 23 May 2010. Available online: http://www.efsa.europa.eu/en/dataclosed/call/ans091123

technical know-how regarding adequate/recommended levels of use in different food applications'. This data provider (Documentation provided to EFSA n. 24) provided three identical levels on meat products. These levels were not considered in the current estimates.

Usage levels from the call for data from 2010[10] were also not used in this opinion, since the food categories on which they were reported for are well covered by the most recent data received.

Appendices A and B present data on the use levels of carrageenan (E 407) and processes Eucheuma seaweed (E 407a) in foods as reported by industry.

Summarised data on concentration levels in food submitted by Member States

In total, only two analytical results were reported to EFSA on carrageenan (E 407) by one country (Germany). These data were on liquid cow milk (FCS 01.1). Foods were sampled in year 2003.

Both analytical results on carrageenan (E 407) were left-censored: not quantified (< LOQ). Complete information on the methods of analysis (e.g. validation) was not made available to EFSA, but both samples were derived from accredited laboratories. The Panel noted that both analytical results were reported in a food category in which carrageenan (E 407) is not authorised for direct addition according to Annex II of Regulation (EC) No 1333/2008. Other authorised uses of carrageenan (E 407) according to Annex III to Regulation (EC) No 1333/2008 (Part 1 and 3) may have resulted in carry-over and its detection in this food category. It should be noted that carrageenan (E 407) was not quantified in these food categories and, therefore, it was not considered for the exposure calculation.

Appendix C shows the analytical results of carrageenan (E 407) in foods as reported by Member States.

3.3.2. Summarised data extracted from the Mintel's Global New Products Database

The Mintel GNPD is an online database which monitors product introductions in consumer packaged goods markets worldwide. It contains information of over two million food and beverage products of which more than 900,000 are or have been available on the European food market. Mintel started covering EU's food markets in 1996, currently having 20 out of its 28 member countries and Norway presented in the Mintel GNPD.[23]

For the purpose of this Scientific Opinion, the Mintel GNPD[24] was used for checking the labelling of products containing carrageenan (E 407) and processed Eucheuma seaweed (E 407a) within the EU's food products, as the Mintel GNPD shows the compulsory ingredient information presented in the labelling of products.

According to Mintel, carrageenan (E 407) is labelled on 16,890 and processed Eucheuma seaweed (E 407a) on 653 foods, drinks and food supplement products published between 2012 and 2017.

Appendix D presents the percentage of the food products labelled with E 407 and E 407a between 2012 and 2017, out of the total number of food products per food sub-categories according to the Mintel food classification.

3.3.3. Food consumption data used for exposure assessment

EFSA Comprehensive European Food Consumption Database

Since 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) has been populated with national data on food consumption at a detailed level. Competent authorities in the European countries provide EFSA with data on the level of food consumption by the individual consumer from the most recent national dietary survey in their country (cf. Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011a). New consumption surveys recently[25] added in the Comprehensive database were also taken into account in this assessment.[14]

The food consumption data gathered by EFSA were collected by different methodologies and thus direct country-to-country comparisons should be interpreted with caution. Depending on the food category and the level of detail used for exposure calculations, uncertainties could be introduced owing to possible subjects' underreporting and/or misreporting of the consumption amounts. Nevertheless,

[23] Missing Bulgaria, Cyprus, Estonia, Latvia, Lithuania, Luxembourg, Malta and Slovenia.
[24] http://www.gnpd.com/sinatra/home/ accessed on 5/10/2016 (up to the date of the work done on the opinion).
[25] Available online: http://www.efsa.europa.eu/en/press/news/150428.htm

the EFSA Comprehensive Database represents the best available source of food consumption data across Europe at present.

Food consumption data from the following population groups: infants, toddlers, children, adolescents, adults and the elderly were used for the exposure assessment. For the present assessment, food consumption data were available from 33 different dietary surveys carried out in 19 European countries (Table 7).

Table 7: Population groups considered for the exposure estimates of carrageenan (E 407) and processed Eucheuma seaweed (E 407a)

Population	Age range	Countries with food consumption surveys covering more than one day
Infants	From 12 weeks up to and including 11 months of age	Bulgaria, Denmark, Finland, Germany, Italy, UK
Toddlers[(a)]	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Denmark, Finland, Germany, Italy, Netherlands, Spain, UK
Children[(b)]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Spain, Sweden, UK
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czech Republic, Denmark, Finland, France, Germany, Italy, Latvia, Netherlands, Spain, Sweden, UK
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Netherlands, Romania, Spain, Sweden, UK
The elderly[(b)]	From 65 years of age and older	Austria, Belgium, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Netherlands, Romania, Sweden, UK

(a): The term 'toddlers' in the EFSA Comprehensive Database corresponds to 'young children' in Regulations (EC) No 1333/2008 and (EU) No 609/2013.

(b): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011a).

Consumption records were codified according to the FoodEx classification system (EFSA, 2011b). Nomenclature from the FoodEx classification system has been linked to the Food Classification System (FCS) as presented in Annex II of Regulation (EC) No 1333/2008, part D, to perform exposure estimates. In practice, FoodEx food codes were matched to the FCS food categories.

Food categories selected for the exposure assessment of carrageenan (E 407) and processed Eucheuma seaweed (E 407a)

The food categories in which the use of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised were selected from the nomenclature of the EFSA Comprehensive Database (FoodEx classification system), at the most detailed level possible (up to FoodEx Level 4) (EFSA, 2011b).

Some food categories or their restrictions/exceptions are not referenced in the EFSA Comprehensive Database and could therefore not be taken into account in the present estimate. This omission may have resulted in an underestimation of the exposure. The food categories that were not taken into account are described below (in ascending order of the FCS codes):

- 2.3 Vegetable oil pan spray;
- 6.4.4 Potato Gnocchi;
- 6.6 Batters;
- 6.7 Pre-cooked or processed cereals, only pre-cooked cereals;
- 8.3.3 Casings and coatings and decorations for meat;
- 12.1.2 Salt substitutes;
- 14.2.5 Mead;
- 14.2.7.2. Aromatised wine-based drinks;
- 14.2.7.3 Aromatised wine-based cocktails.

For the following food categories, the restrictions/exceptions which apply to the use of Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) could not be taken into account, and therefore, the whole food category was considered in the exposure assessment. This may result in an overestimation of the exposure:

- 5.1 Cocoa and cocoa products, only energy-reduced or with no added sugar;
- 5.2 Other confectionery including breath refreshening microsweets, except in jelly mini-cups, defined, for the purpose of this Regulation, as jelly confectionery of a firm consistence, contained in semi rigid mini-cups or mini-capsules, intended to be ingested in a single bite by exerting pressure on the mini-cups or mini-capsule to project the confectionery into the mouth;
- 7.1 Bread and rolls, except products in 7.1.1 and 7.1.2;
- 8.2 Meat preparations as defined by Regulation (EC) No 853/2004, only preparations in which ingredients have been injected; meat preparations composed of meat parts that have been handled differently: minced, sliced or processed and that are combined together. Except bifteki, soutzoukaki, kebap gyros and souvlaki;
- 8.3.2 Heat-treated meat products, except foie gras, foie gras entier, blocs de foie gras, Libamáj, libamáj egészben, libamáj tömbben;
- 9.3 Fish roe, only processed fish roe.

The FCs 17.1/17.2/17.3 Food supplements, in solid, liquid, syrup-type or chewable form, the form cannot be differentiated and the same use level was applied to the whole FC 17.

Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are also allowed in FC 13.2, 13.3 and 13.4. Food items under food categories 13.2, 13.3 and 13.4 consumed by population groups- children, adolescents, adults and the elderly- may be very diverse and, in addition, there is very limited information on their consumption. Therefore, eating occasions belonging to the food categories 13.2, 13.3 and 13.4 were reclassified under food categories in accordance to their main component.

The use levels available for food categories 13.2, and 13.3 (there were no reported data for FC 13.4) were not considered for the exposure assessment.

Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are also authorised in FC 18 (Processed foods not covered by categories 1 to 17, excluding foods for infants and young children). Considering that FC 18 is extremely unspecific (e.g. composite foods, processed foods, prepared or composite dishes belonging to this food category were reclassified under food categories in accordance to their main component and included as such in the exposure assessment). The use levels available for food categories 18 were not considered for the exposure assessment.

No information on the usage levels of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) has been reported to EFSA for 19 food categories and therefore these could not be taken into account (Appendix E). This may have resulted in an underestimation of the exposure if E 407 and E 407a are used in these food categories. Comparing these food categories with the data extracted from the Mintel database (information on the use of E 407/E 407a was found for the categories 04.2.5.3, 06.3, 06.4.2, 06.5, 12.3, 14.1.3, 15.1), the Panel noted that the use of carrageenan and processed Eucheuma seaweed in these food categories was limited.

Overall, for both the maximum level exposure and for the refined scenarios 41 food categories were included, in the present exposure assessment to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) (Appendix E).

3.4. Exposure estimate

3.4.1. Exposure to carrageenan E 407 and to processed Eucheuma seaweed E 407a from their use as food additives

The Panel estimated chronic exposure for the following population groups: infants; toddlers, children, adolescents, adults and the elderly. Based on information from the Mintel's GNPD, the Panel considered that E 407 and E 407a are not likely to be used in combination in the same food product and, therefore, the exposure assessment of E 407 and E 407a was performed considering the reported use level for either E 407 and E 407a independently per food category (Appendix D). Dietary exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) was calculated by multiplying carrageenan (E 407) and processed Eucheuma seaweed (E 407a) concentrations for each food category (Appendix E) with their respective consumption amount per kilogram of body weight for

each individual in the Comprehensive Database. The exposure per food category was subsequently added to derive an individual total exposure per day. These exposure estimates were averaged over the number of survey days, resulting in an individual average exposure per day for the survey period. Dietary surveys with only one day per subject were excluded as they are considered as not adequate to assess repeated exposure.

This was carried out for all individuals per survey and per population group, resulting in distributions of individual average exposure per survey and population group (Table 7). Based on these distributions, the mean and 95th percentile of exposure were calculated per survey for the total population and per population group. High percentile exposure was only calculated for those population groups where the sample size was sufficiently large to allow calculation of the 95th percentile of exposure (EFSA, 2011a). Therefore, in the present assessment, high levels of exposure for infants from Italy and for toddlers from Belgium, Italy and Spain were not included.

Exposure assessment to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) was carried out by the ANS Panel based on two different sets of concentration data: (1) MPLs as set down in the EU legislation or maximum levels of data provided to EFSA when E 407 and E 407a are authorised at QS (defined as the *regulatory maximum level exposure assessment scenario*); and (2) reported use levels (defined as the *refined exposure assessment scenario*). These two scenarios are discussed in detail below.

These scenarios do not consider the consumption of food supplements (FC 17.1, 17.2 and FC 17.3) or FSMP which are covered in added scenarios detailed below.

A possible additional exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives authorised in accordance with Annex III to Regulation (EC) No 1333/2008 was not considered in any of the above exposure assessment scenarios.

In addition, formaldehyde exposure was calculated for the non-brand-loyal scenario.

Maximum level exposure assessment scenario

The regulatory maximum level exposure assessment scenario is based on the MPLs as set in Annex II to Regulation (EC) No 1333/2008 and listed in Table 6. As carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised according to QS in almost all food categories, a 'maximum level exposure assessment' scenario was estimated based on the maximum reported use levels provided by industry, excluding exposure via food supplements and FSMP, as described in the EFSA Conceptual framework (EFSA ANS Panel, 2014).

The Panel considers the exposure estimates derived following this scenario as the most conservative since it is assumed that the consumer will be continuously (over a lifetime) exposed to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) in food at the maximum reported use levels.

Refined exposure assessment scenario

The refined exposure assessment scenario is based on use levels reported by industry. This exposure scenario can consider only food categories for which the above data were available to the Panel.

Appendix E summarises the concentration levels of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) used in the refined exposure assessment scenario. Based on the available data set, the Panel calculated two refined exposure estimates based on different model populations:

- The brand-loyal consumer scenario: It was assumed that a consumer is exposed long-term to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) present at the maximum reported use level for one food category. This exposure estimate is calculated as follows:
  - Combining food consumption with the maximum of the reported use levels or the maximum of the analytical results, whichever was highest or available, for the main contributing food category at the individual level.
  - Using the mean of the typical reported use levels or the mean of analytical results, whichever was highest or available, for the remaining food categories.
- The non-brand-loyal consumer scenario: It was assumed that a consumer is exposed long-term to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) present at the mean reported use levels in food. This exposure estimate is calculated using the mean of the typical reported use levels or the mean of analytical results for all food categories.

Specific exposure assessment scenarios

- **Food supplement consumers only scenario**

Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised in the food category 17 Food supplements as defined in Directive 2002/46/EC excluding food supplements for infants and young children. As exposure via food supplements may deviate largely from the one via food, and that the number of food supplement consumers may be low depending on populations and surveys, an additional scenario was calculated in order to reflect additional exposure to food additives from food supplements compared to exposure to food additives excluding these sources. This scenario will be estimated as follow:

- Consumers only of food supplements were assumed to be exposed to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) present at the maximum reported use level on a daily basis via consumption of food supplements.
- For the remaining food categories, the mean of the typical reported use levels was used.

As food category 17 does not consider food supplements for infants and toddlers, as defined in the legislation, exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from food supplements was not estimated for these two population groups.

- **FSMP consumers only scenario**

As carrageenan (E 407) is also authorised in the food categories 13.1.5.1 and 13.1.5.2 with MPL, an additional exposure assessment scenario taking into account these two food categories was performed to estimate the exposure of infants and toddlers who may eat and drink these foods for special medical purposes (FSMP).

The consumption of these foods is not reported in the EFSA Comprehensive database. To consider potential exposure to carrageenan (E 407) via these foods, the Panel assumes that the amount consumed of FSMP in infants and toddlers resembles that of comparable foods in infants and toddlers from the general population. Thus, the consumption of FSMP categorised as food category 13.1.5 is assumed to equal that of formulae and food products categorised as food categories 13.1.1, 13.1.2, 13.1.3 and 13.1.4.

FSMP consumed in other population groups (children, adolescents, adults and the elderly) may be very diverse; they were not considered because of very limited information on consumption. Eating occasions belonging to the food categories 13.2, 13.3, 13.4 were therefore reclassified under food categories in accordance to their main component and no exposure estimates for these population groups were calculated.

This scenario was estimated as follows:

- Consumers only of FSMP were assumed to be exposed to carrageenan (E 407) present at the maximum reported use level (data provided by food industry, reported values equal to the MPL) on a daily basis via consumption of the food categories 13.1.5.1 and 13.1.5.2 (infant formulae, follow-on formulas and processed cereal-based foods and baby foods for infants and young children as defined by Commission Directive 2006/125/EC).
- For the remaining food categories, the mean of the typical reported use levels was used.

Dietary exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a)

Table 8 summarises the estimated exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives in six population groups (Table 7) according to the different exposure scenario's. Detailed results per population group and survey are presented in Appendix G.

Table 8: Summary of anticipated exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives in the maximum level exposure assessment scenario and in the refined exposure scenarios, in six population groups (minimum–maximum across the dietary surveys in mg/kg bw per day)

	Infants (4–11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (≥ 65 years)
Maximum level exposure assessment scenario						
Mean	21.8–86.7	135.3–581.4	109.6–487.0	63.5–264.6	34.7–138.6	24.7–89.4
95th percentile	64.9–324.8	354.3–913.2	245.0–999.1	152.7–534.7	92.5–326.8	58.1–175.0
Refined estimated exposure assessment scenario						
Brand-loyal scenario						
Mean	15.1–64.3	99.4–374.9	70.1–296.9	38.9–175.9	22.0–88.9	14.2–50.8
95th percentile	42.4–256.9	262.5–758.6	143.8–690.8	103.8–397.2	63.3–247.2	29.8–116.8
Non-brand-loyal scenario						
Mean	5.2–18.7	19.2–70.7	18.3–57.8	9.5–27.1	5.0–16.3	4.4–15.6
95th percentile	19.4–49.0	55.0–121.6	36.0–109.8	17.8–50.3	10.2–34.7	8.4–27.5

From the maximum level exposure assessment scenario, the mean exposure to Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives ranged from 21.8 mg/kg bw per day in the infants to 581.4 mg/kg bw per day in toddlers. The 95th percentile of exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) ranged from 58.1 mg/kg bw per day in the elderly to 999.1 mg/kg bw per day in children.

From the refined estimated exposure scenario, in the brand-loyal scenario, mean exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives ranged from 14.2 mg/kg bw per day in the elderly to 374.9 mg/kg bw per day in toddlers. The high exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) ranged from 29.8 mg/kg bw per day in the elderly to 758.6 mg/kg bw per day in toddlers. In the non-brand-loyal scenario, mean exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives ranged from 4.4 mg/kg bw per day in the elderly to 70.7 mg/kg bw per day in toddlers. The 95th percentile of exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) ranged from 8.4 mg/kg bw per day in the elderly to 121.6 mg/kg bw per day in toddlers.

From the exposure scenario taking into account the foods for special medical purposes, for consumers only, mean exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives ranged from 2.5 mg/kg bw per day to 30.9 mg/kg bw per day for infants and from 0.3 mg/kg bw per day to 19.4 mg/kg bw per day for toddlers. The 95th percentile of exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) for infants ranged from 21.9 mg/kg bw per day to 49.4 mg/kg bw per day and for toddlers from 9.8 mg/kg bw per day to 33.5 mg/kg bw per day.

From the refined estimated exposure scenario taking into account the consumption of food supplements, for consumers only, among children, adolescents, adults and the elderly, mean exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives ranged from 9.9 mg/kg bw per day in adults to 77.9 mg/kg bw per day in children. The 95th percentile of exposure ranged from 23.9 mg/kg bw per day in the elderly to 88.0 mg/kg bw per day in children (see Appendix H).

Main food categories contributing to exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) using the maximum level exposure assessment scenario

From the maximum level exposure assessment scenario, the main contributing food categories to the total mean exposure estimates for infants were flavoured fermented milk products and fine bakery wares. For toddlers, children and adolescents, the main contributing food categories were flavoured fermented milk products, fine bakery wares and flavoured drinks; while, for adults and the elderly, the main contributing food categories were fine bakery wares and flavoured drinks.

The main food categories contributing to the combined exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) using the maximum level exposure assessment scenario are presented in Appendix G.

Main food categories contributing to exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) using the refined exposure assessment scenario

In the brand-loyal scenario, the main contributing food categories were flavoured fermented milk products and fine bakery wares for infants; for toddlers, children and adolescents the main contributing food categories were flavoured fermented milk products, fine bakery wares and flavoured drinks. For adults and elderly, the main contributing food categories were fine bakery wares and flavoured drinks.

In the non-brand-loyal scenario, the main contributing food categories were: flavoured fermented milk products and processed fruit and vegetables for infants; flavoured fermented milk products and fine bakery wares for toddlers and children; fine bakery wares for adolescents, adults and the elderly.

The main food categories contributing to the combined exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) using the refined exposure scenarios are presented in Appendix G.

Exposure to formaldehyde from the occurrence in carrageenan (E 407) and processed Eucheuma seaweed (E 407a)

According to the EU specifications, the content of formaldehyde is limited to a maximum of 5 mg/kg in carrageenan products. Information from literature suggested that residual levels of formaldehyde in five carrageenan products tested using the method provided by one of the interested parties (Documentation provide to EFSA n. 48) are in the range 0.5–1.5 mg/kg.

Based on these values, the Panel calculated the exposure to formaldehyde from the occurrence of carrageenan products as food additives in different foodstuffs for the highest exposure estimates in toddlers based on the refined brand loyal exposure scenario. The highest P95th exposure to residuals levels of formaldehyde was 3.8 μg/kg bw per day using the EU specification of 5 mg/kg down to 1.14 μg/kg bw per day using the highest residual levels of 1.5 mg/kg reported in Documentation provide to EFSA n. 48.

The Panel evaluated the risks associated with methanol and its subsequent metabolism to formaldehyde in the opinion on aspartame (EFSA ANS Panel, 2013). Because the increase of formaldehyde above the baseline level due to 4 mg formaldehyde/kg bw per day was a small fraction of the natural occurring variation, the Panel concluded that this level of formaldehyde did not constitute a significant additional risk. The Panel noted that the exposure at the limit of specifications for formaldehyde in carrageenan products was 3.8 μg/kg bw per day which is around 1,100 times lower than the amount arising from aspartame.

Therefore, the Panel concurred with the previous EFSA AFC opinion (EFSA, 2007) that exposure to carrageenan as additives containing residual formaldehyde, at the EU purity criteria level of 5 mg/kg, would be of no safety concern.

Uncertainty analysis

Uncertainties in the exposure assessment of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) have been discussed above. In accordance with the guidance provided in the EFSA opinion related to uncertainties in dietary exposure assessment (EFSA, 2007), the following sources of uncertainties have been considered and summarised in Table 9.

Table 9: Qualitative evaluation of influence of uncertainties on the dietary exposure estimate

Sources of uncertainties	Direction[a]
Consumption data: different methodologies/representativeness/underreporting/misreporting/no portion size standard	+/–
Use of data from food consumption survey covering only a few days to estimate long-term (chronic) exposure for high percentiles (95th percentile)	+
Correspondence of reported use levels and analytical data to the food items in the EFSA Comprehensive Food Consumption Database: uncertainties to which types of food the levels refer to	+/–

Sources of uncertainties	Direction[a]
Food categories selected for the exposure assessment: exclusion of food categories due to missing FoodEx linkage (n = 9 out of 79 food categories)	–
Food categories selected for the exposure assessment: inclusion of food categories without considering the restriction/exception (n = 7 out of 79 food categories)	+
Food categories selected for the exposure assessment: data not available for certain food categories which were excluded from the exposure estimates (n = 19 out of 79 food categories)	–
Foods which may contain the food additive according to Annex III to Regulation (EC) No 1333/2008 not taken into account	–
Maximum level exposure assessment scenario: • exposure calculations based on the maximum reported use levels (reported use from industries)	+
Refined exposure assessment scenarios: • scenario including only the food categories according to Annex II to Regulation (EC) No 1333/2008 • exposure calculations based on the maximum or mean levels (reported use from industries)	– +/–
Food supplements consumers only scenario: • exposure calculations based on consumers only • exposure calculations based on the mean levels (reported use from industries for all foods except food supplements	+ –/+
FSMP, consumers only scenario: • exposure calculations based on consumers only • exposure calculations based on the MPL levels for the FSMP and mean reported use levels for all other foods • foods which may contain the food additive according to Annex III to Regulation (EC) No 1333/2008 not taken into account	+ + –
Uncertainty in possible national differences in use levels of food categories	+/–

(a): +, uncertainty with potential to cause over-estimation of exposure; –, uncertainty with potential to cause underestimation of exposure.

Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised as a Group I food additive in 79 food categories and have specific authorised uses in six other categories (Table 6). Since the majority of food categories correspond to the general Group I food additives authorisation, carrageenan (E 407) and processed Eucheuma seaweed (E 407a) may not necessarily be used in some of these food categories. This may explain why use levels for carrageenan (E 407) and processed Eucheuma seaweed (E 407a) were not reported by the food industry for 19 food categories. However, the Panel noted that information from the Mintel GNPD (Appendix D) indicated that seven of these 19 food categories were labelled with carrageenan and processed Eucheuma seaweed (fruit and vegetable spreads, breakfast cereals, noodles etc.).

Overall, the Panel considered that the uncertainties identified in both the maximum level exposure scenario and the refined scenario would, in general, result in an overestimation of the real exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives in European countries considered in the EFSA European database. This consideration is based on the assumption that the food additive is not used in those food categories in which it is permitted but for which no usage data were provided by the stakeholders (see Annexes A.1 and A.2).

For FSMP, the Panel noted that one industry reported a use of carrageenan as food additive at the level of the regulation in one niche product which is not aligned with information reported from the Mintel GNPD (Appendix E) where no FSMP products seems labelled on the market. If this information is confirmed, it means that uncertainties arising from the FSMP exposure scenario would lead to an overestimate of the current exposure to carrageenan products as food additives calculated by the Panel.

3.4.2. Exposure via other sources

Pharmaceutical and other uses

Information on pharmaceutical uses was obtained by searches of the literature, the websites of national competent authorities for medicinal products and publicly available SmPC (summary of product characteristics) on the nationally available authorised products indicated to EFSA by the European Medicines Agency (EMA) communication (Documentation provided to EFSA n. 3).

Carrageenan is used in pharmaceutical products as well-known excipient, e.g. as pseudo-emulsifier or as thickening and gelling agent (Teuscher, 1997). It is used in solid, semi-solid and liquid dosage forms (Blaschek et al., 2016).

In former times, carrageenan was used as mucilage in cases of diarrhoea and as bulk-foaming laxative to treat obstipation. The dosage was given with 1.5 g herbal substance/cup of water as decoction. Gastrointestinal discomfort including gas in the intestine is mentioned as possible undesired effect when carrageenan is taken in very high amounts, as for all bulk forming substances (Blaschek et al., 2014; Martindale, 2014).

Carrageenan is used in some countries in combination products (Martindale, 2014).

For carrageenan as an active ingredient, only few authorised medicinal combination products exist within the EU but not for oral use.

There is no additional information available regarding any use of processed Eucheuma seaweed.

3.5. Biological and Toxicological data

In the present opinion, the term 'carrageenan' is used when the test compound in the biological and toxicological studies has been described by the authors as 'native carrageenan' or 'undegraded carrageenan' or 'conventionally processed carrageenan'. For the degraded carrageenan, the terms used by the authors of the studies, e.g. poligeenan, C16, were reported.

Taking into account that the studies performed with carrageenan date from back to the 1940s up to present as well as the fact that the source description has changed during this time and differs between authors (e.g. Weiner et al., 2017), the Panel decided, that, an interpretation of the identity (expressed in type(s) of carrageenan) was necessary for each study in order to make it possible to compare the results of different studies. An explanation of how this has been performed is in Appendix J.

The Panel noted that in most studies no adequate information on the molecular weight distribution of carrageenan was available, and therefore it was in most cases not possible to assess if the carrageenan tested complied with the EU specifications (the limit of low molecular weight carrageenan (below 50 kDa) of 5%).

As mentioned above, the information on the molecular weight distribution in the tested individual carrageenan preparations is often missing or unprecise mixing the terms 'molecular weight', 'weight-average molecular weight' and 'number-average molecular weight'. In this opinion, the term describing the molecular weight of the carrageenan preparation tested, is used as reported by the authors of the studies.

The Panel further noted that according to Weiner (2014, 2016), the vehicle employed to administer carrageenan was critical for understanding the toxicological effects and their interpretation because the conformation of the carrageenan molecule changes depending on the vehicle. Most animal studies administered carrageenan in diet or in drinking water and some studies were conducted by oral gavage in water. When carrageenan was dissolved in water at dilute concentration (50.1%) with no added potassium or calcium ions, dietary solids or protein, the polymer independent of type exists in a disorganised random conformation (Blakemore and Harpel, 2010). Under these conditions, which are not representative of the use of the food additive E 407, all the carrageenan molecules are available for maximum interaction with the gastrointestinal (GI) tract cells.

3.5.1. Absorption, distribution, metabolism and excretion of Carrageenan

No data were available on absorption, distribution, metabolism and excretion (ADME) of processed Eucheuma seaweed. The Panel noted that in a review, it was concluded that there was no reason to expect processed Eucheuma seaweed to behave differently than carrageenan, based on their chemical structure (Cohen and Ito, 2002).

In vitro studies

The rate of degradation of eight batches of κ-carrageenan (Copenhagen Pectin Factory Ltd., Denmark, interpreted by the Panel with a ratio ι:κ:λ type 0:100:0) in simulated gastric juice (at pH 1.2 or 1.9) was determined by Ekstrom (1985). The various batches of κ-carrageenan used were similar in terms of weight-average molecular weight (167 ± 7 kDa) and shape of the distribution curve of the molecular weight. An average of 25% of the carrageenan in the batches had a mass around 100 kDa and 9% less than 50 kDa. After 2 h in simulated gastric juice at pH 1.2, almost 90% of the carrageenan had a mass less than 100 kDa and 25% had a mass less than 20 kDa. At pH 1.9, the rate of degradation was lower; after 2 h, 65% of the carrageenan had a mass less than 100 kDa and 10% than 20 kDa. The presence of pepsin did not affect the rate of the degradation although carrageenan has a strong protein-binding tendency. According to the author, the acidity and the rate of passage through the stomach will determine the degree of degradation of carrageenan.

Capron et al. (1996) investigated the stability of κ- and ι-carrageenan (Sanofi, interpreted by the Panel with a ratio ι:κ:λ type 0:100:0 and ι:κ:λ type 100:0:0) to depolymerisation in batch experiments (for 3 or 6 h at pH 1.2, 2, 3 or 8) or using an artificial stomach. Degradation was measured by using SEC and multiangle laser LS data. No hydrolysis of κ-carrageenan was observed at pH 8. Under the most drastic conditions (6 h, pH 1.2), the weight-average molecular weight remains higher than 200 kDa and only 20% has a molecular weight less than 100 kDa. Carrageenan in helical form (with potassium) was less susceptible to acid hydrolysis whereas ι-carrageenan was more resistant than the κ-type. Moreover, in conditions mimicking gastric digestion using simulated gastric juice, the formation of fragments of low molecular weight (less than 100 KDa) from well-defined food-grade carrageenan (E 407) is quite limited (less than 10%). The Panel noted that these results are in contrast to the previous findings of Ekstrom (1985) for which a quite complete degradation of κ-carrageenan was observed in solutions of low pH.

A total of 154 strains from 22 species of *Bifidobacterium, Peptostreptococcus, Lactobacillus, Ruminococcus, Coprococcus, Eubacterium a*nd *Fusobacterium*, which occur in high concentrations in the human colon, were tested for their ability to ferment 21 different complex carbohydrates. Among them, carrageenan (unspecified type; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) was not fermented by any strains of the investigated species (Salyers et al., 1977).

Fermentations of 10 polysaccharides including carrageenan (unspecified type; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30), by 24 species of the family Enterobacteriaceae (Klebsielleae and other gram-negative facultative bacilli) were examined by Ochuba and von Riesen (1980). Carrageenan was fermented by 15 species including *Klebsiella* (*K. oxytoca* and *K. pneumoniae*), *Aeromonas*, *Enterobacter*, *Pectobacterium*, *Serratia* and Vibrio strains. According to the authors, this study demonstrated the possible fermentation of carrageenan by enteric bacteria.

In vivo studies

Rats

In groups of three to seven male and female rats (no further information about the strain) fed with 2, 5, 10, 15, or 20% (about 2,000–20,000 mg/kg bw per day) of κ/λ-carrageenan from *C. crispus* (commercial product – Seakem, type 6, from Marine Colloids, Rockland, Maine; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio ι:κ:λ type 0:70:30, 'molecular weight distribution not indicated') over 23–148 days via the diet, more than 90% of the ingested carrageenan was excreted via faeces (Hawkins and Yaphe, 1965).

A comparable result was obtained in groups of five young male Charles River CD rats fed with 0% and 5% (equivalent to 6,000 mg/kg bw per day) of ι-carrageenan (commercial product from Auby, Neuilly-sur-Seine, France; interpreted by the Panel as isolated from *E. spinosum* with an approximate ratio ι:κ:λ type 100:0:0, weight-average molecular weight: 360–540 kDa) (Dewar and Maddy, 1970) or 5% of degraded carrageenan (C16, degraded ι-carrageenan, prepared from *E. spinosum* seaweed, commercial product from Glaxo, Paris, with weight-average molecular weight between 20 and 30 kDa) over 10 days. The faecal levels of carrageenan or degraded carrageenan were calculated from the excreted quantities of 3,6-anhydrogalactose. The treatment had no adverse effects on feed intake. Faecal excretion of carrageenan amounted to 80% of the administered dose of native carrageenan and faecal excretion of degraded carrageenan amounted to 86% of the administered dose of degraded carrageenan (C16). According to the authors, the low value for the percentage of carrageenan

excreted might have been due to inaccuracy or errors in measuring the daily feed intake and in assessing the background level of the 3,6-anhydrogalactose content of the faeces from rats on control diet.

Studies conducted with Sprague–Dawley rats fed diets containing 4% κ/λ-carrageenan (commercial product calcium carrageenan, Sea Kem, type II, Marine Colloids, Co.; interpreted by the Panel as *C. crispus* with an approximate ratio ι:κ:λ type 0:70:30, 'molecular weight distribution not indicated'), separate absorption studies were done with adult males (Tomarelli et al., 1974). The animals (n = 8) were placed in metabolism cages, fed a basal diet for 7 days, followed by 5 days of feeding the carrageenan diet, and then 2 more days on the basal diet. By using an improved colorimetric method, 99% of the ingested carrageenan was found to be excreted via faeces.

A study including different trials was performed in rats by Pittman et al. (1976). In a first trial, Sprague–Dawley rats of both sexes (n = 3) were given carrageenan from *E. spinosum* (number-average molecular weight: 8.7–145 kDa, ratio ι:κ:λ type 100:0:0, Marine Colloids, Co) in distilled water at a dose of 500 mg/kg bw per day via gavage daily for 9 months. At the end of this time, rats were killed and the livers were retained for analysis. In another experiment weanling Sprague–Dawley rats of both sexes (n = 5) were fed control diet or a diet containing 5% (about 5,000 mg/kg bw per day) of carrageenan from *C. crispus* (number-average molecular weight: 186–214 kDa ratio ι:κ:λ types 0:71:29 to 0:88:12, Stauffer Chemicals, Inc). After 13 weeks, five animals of each group and sex were killed and samples of liver were analysed for carrageenan. Faecal samples were also collected for analysis. Faecal and liver samples were examined qualitatively by gel electrophoresis, and quantitative measurements of carrageenan were carried out on samples of liver and urine. For the high molecular carrageenan (> 100 kDa) no or only little absorption was noted based on the absence of carrageenan from the livers of rats. By contrast, evidence for the presence of carrageenan in the livers of rats was obtained only in those animals given ι-carrageenan with number-average molecular weight of 88 kDa or less, for 9 months. The Panel noted that low molecular carrageenan was partly absorbed in rats given dietary ι-carrageenan.

Chen et al. (1981) used a gel electrophoresis method developed for the detection of carrageenan (unspecified type; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30, 'molecular weight distribution not indicated') in rat liver homogenates (measurable limit of 15 μg carrageenan/g liver tissue). No carrageenan was found in the livers of rats fed 25% (about 25,000 mg/kg bw per day) over one month. Further details were not reported.

In the subchronic study of Abraham et al. (1985), three groups of 30 Sprague–Dawley rats were exposed to 1% or 5% carrageenan (commercial product *Iridaea* carrageenan from Hercules Inc., Copenhagen Pectin Fabrik Ltd., Denmark; interpreted by the Panel as isolated from *Iridaea ciliate* = *Sarcothalia crispata,* mixture of κ and λ, 'molecular weight distribution not indicated') via diet (equivalent to 900 and 4,500 mg/kg bw per day or with cellulose at a 5% dietary level (13 males and 17 females) for 13 weeks. Storage of carrageenan or carrageenan-like material was investigated by the presence of metachromatic material following toluidine blue staining, increase in lysosomal enzyme activity in sites similar to ultrastructural localisation of fibrillar material and chemical analysis. According to the authors, this study showed no retention or storage of native carrageenan in the gut or liver of chronically administered rats. The absence of tissue retention of native carrageenan was also observed in an additional chronic experiment in which two strains of rats received this carrageenan for 39 weeks.

Arakawa et al. (1986, 1988) found that dietary κ-carrageenan with a molecular weight > 100 kDa (commercial product of κ-carrageenan CS-47, from San-ei Chemical Ind., Co., Osaka; interpreted by the Panel from *E. cottonii* or other sources with an approximate ratio ι:κ:λ type 0:100:0) was not metabolised to lower molecular weight material in the faeces of rats and that 97–98% of the ingested carrageenan dose (4 g/kg) was excreted in the faeces as unchanged carrageenan.

Nicklin et al. (1988) investigated the fate of radiolabelled ^{3}H-ι-carrageenan in rats (n = 3) administered by gavage in water (50 μg/mL per rat). ι-Carrageenan (commercial ι-carrageenan from Hercules Ltd., UK; interpreted by the Panel with an approximate ratio ι:κ:λ type 100:0:0, 'molecular weight distribution not indicated') was incubated with galactose oxidase and tritiated borohydride, leading to a tritiated molecule labelled on CH_2OH group. Some radioactivity was observed in the intestinal wall, Peyer's Patches, mesenteric lymph nodes, caecal lymph nodes, and serum. Autoradiographs of tissues indicated that the radioactivity would be associated with the macrophages. The authors suggested that carrageenan could enter the body via the macrophages of the Peyer's Patches and caecal lymph nodes. The majority of the radioactivity was excreted in the faeces.

However, according to the authors, exchange of the tritium label could occur with the hydrogen of body water. The Panel considered this study of low relevance.

Conventional rats with normal gut microbiota and rats (n = 10) with their gut microbiota replaced by human fecal microbiota were given 2.5% κ-carrageenan (commercial product of κ-carrageenan CS-47, from Sigma, Type 1, C-1013, batch 86F0698; interpreted by the Panel from *E. cottonii* or other sources with an approximate ratio ι:κ:λ type 0:100:0) in water as a gel (Taché et al., 2000). Faecal samples were collected and then heated at 80°C for 30 min in water (pH 8) and centrifuged. The molecular weight of carrageenan in faeces was measured in supernatants by SEC consisting of an HPLC system equipped with a multiangle laser LS detector providing the absolute molecular weight of the eluted polymers. No carrageenan was detected in faecal samples from untreated control rats. The molecular weight of steam-heated dietary carrageenan ranged from 310 to 320 KDa. In treated rats, the average molecular weights of the carrageenan found in faeces were 346 KDa and 307 KDa in conventional rats and in rats with human faecal microbiota, respectively. This slight difference was not statistically significant. According to the authors, the average molecular weight of carrageenan was not changed to a great extent during its passage through the GI tract.

Food-grade λ-carrageenan (information given in Japanese interpreted by the Panel as from *Chondrus* or *Gigartina* sp. with a ratio ι:κ:λ unknown) was included in the diet of rats at a level of 5% (Uno et al., 2001b). After an overnight deprivation of food, animals (one male and one female) were given access to the treated diet for one day and then to the untreated basal diet for 2 days. Faeces were collected for each rat at three time points each day and analysed by using GPC to separate high and low molecular weight λ-carrageenan components in faeces. ICP atomic emission spectroscopy (ICP-AES) was used for direct detection of the sulfur of the carrageenan present in faeces. The molecular weights of the carrageenan found in the faeces after day 1 and day 2 were similar to that of the carrageenan in the blended diet: 782 kDa in faeces compared to 832 kDa in diet. No carrageenan was detected in faeces excreted on day 3. According to the authors, dietary λ-carrageenan was excreted in the faeces without any decomposition to lower molecular weight fractions.

Guinea pigs

In the study of Pittman et al. (1976), female guinea pigs were divided into groups (n = 6) and fed control diet or a diet containing 2% (500–1,000 mg/kg bw per day) of carrageenan from *E. spinosum* (number-average molecular weight of 5–145 kDa, ratio ι:κ:λ type 100:0:0, Marine Colloids Inc. or Glaxo Lab.) for a period of 7–10 weeks after which they were killed and samples of livers were obtained for analysis. In two later experiments, other groups of female guinea pigs (n = 3) were given by gavage, carrageenan from *E. spinosum* with number-average molecular weight ranging from 5–145 kDa (ratio ι:κ:λ type 100:0:0, Marine Colloids Inc.) or from *C. crispus* (number-average molecular weight of 8.5–314 kDa, ratio ι:κ:λ types 0:100;0; 0:0:100 or 0:70:30, Marine Colloids Inc.) as 1% solutions (about 1,000–2,000 mg/kg bw per day) via drinking water for 2 weeks. At the end of the first of these latter experiments, samples of liver, faeces and bladder urine were analysed, while at the end of the second experiment samples of liver and faeces were examined. In all trials, faecal and liver samples were examined qualitatively by gel electrophoresis, and quantitative measurements of carrageenan were carried out on samples of liver and urine. For the high molecular weight carrageenan (> 100 kDa), no or only little absorption was noted based on the absence of carrageenan in the livers or the urine of guinea pigs. By contrast, significant amounts of carrageenan were found in livers of animals given carrageenan with number-average molecular weight lower than 88 kDa, whatever the type of carrageenan was. According to the authors, urinary excretion of carrageenan was limited to low-molecular-weight material (number-average molecular weight: < 20 kDa). Qualitative and quantitative evidence indicated that there was an upper limit to the size of carrageenan molecules absorbed, but estimates of this upper limit ranged from 10 to 85 kDa depending upon the analytical approach. Absorption of low molecular carrageenan from the drinking-water was generally higher than following dietary administration. The Panel noted that low molecular carrageenan would be partly absorbed, particularly when administered by gavage and given via drinking water.

Minipigs and pigs

The studies in neonatal minipigs and in neonatal pig (Documentation provided to EFSA n. 32; Documentation provided to EFSA n. 33; also described in Weiner et al., 2015) employed a liquid chromatography with tandem mass spectrometry (LC–MS/MS) analytical method developed using 'poligeenan' as a surrogate for detection of the low molecular weight carrageenan in blood.

In the neonatal minipig study (Gottingen minipigs) (Documentation provided to EFSA n. 32), following daily oral administration of carrageenan (the test compound is assumed to be identical with that described below in the study by Weiner et al., 2015) at doses of 0, 300 or 3,000 mg/kg formula (equal to 60–66 or 600–666 mg/kg bw per day) for 10 days, starting on postnatal day (PND) 2, there were no quantifiable serum poligeenan concentrations in animals receiving the 600–666 mg/kg bw per day on both evaluation days. According to the authors, the unique reported values for two animals (one control and one receiving 60–66 mg/kg bw per day) may represent interfering compounds rather than actual exposure to low molecular weight carrageenan.

In the study in neonatal pigs (Domestic Yorkshire Crossbred Swine) (Documentation provided to EFSA n. 33; Weiner et al., 2015), the test material sample of κ/γ-carrageenan (FMC Lot. 90303011) (ratio κ/λ unknown) was fully characterised for molecular weight, percentage of molecular weight below 50 kDa, known as LMT as per European requirements. The weight-average molecular weight of the test material sample was 664–732 kDa with a LMT of 0.3–3.9%. The test substance was administered at doses of 0, 300, 1,000 or 2,250 mg/kg formula for 28 days starting on PND 3 (highest dose equal to 430–448 mg/kg bw per day). The authors reported an analytical signal in male piglets, corresponding to that expected for the low molecular weight carrageenan. According to the author, this pattern was not consistent with the expected exposure based on the calculated compound consumption of carrageenan. This is supported by the fact that (1) a positive signal was elicited in all males in the control group; (2) similar values were observed in all treated males at 300, 1,000, and 2,250 mg/kg; (3) the signal did not produce a dose response effect in the treated groups (300, 1,000 and 2,250 mg/kg) and (4) a positive signal was almost exclusively limited to male animals. These data would indicate that the signal in the plasma samples is not a low molecular carrageenan.

Considering the study in neonatal minipigs and neonatal pigs, the Panel noted the use of appropriate animal models for representing the immature gastrointestinal tract in human infants. However, due to problems in the outcomes of analytical assays, the Panel was unable to conclude on a possible *in vivo* degradation of carrageenan.

Primates

In the short-term study of Abraham et al. (1972), male and female rhesus monkeys (*Macaca mulatta,* n = 10) were given a type of κ/λ-carrageenan derived from *C. crispus* (carrageenan mixture of κ and λ with number-average molecular weight of approx. 800 kDa obtained from Marine Colloids, Inc. Rockland, ME, Lot. Beta282400; interpreted by the Panel as *C. crispus* with an approximate ratio ι:κ:λ type 0:70:30) via drinking water as a 1% solution (about 1,300 mg/kg bw per day) for 7–11 weeks. There was no evidence of carrageenan storage by contrast to the administration of degraded ι-carrageenan (C16) (prepared by hydrolysis of an extract of *E. spinosum* (C16), provided by Laboratoires Glaxo, Paris, with the weight-average molecular weight in the ranges 20–30 kDa) which produced extensive reticuloendothelial retention of degraded material that was still present in Kupffer cells 6 months after administration of C16 was stopped.

In the long-term toxicity study (Documentation provided to EFSA n. 34), rhesus monkeys (19 males and 21 females) were divided into four groups with 10 animals each and dosed with 0, 50, 200, 500 mg/kg bw per day κ/λ-carrageenan (commercial product Gelcarin HMR from Marine Colloids, Rockland, Maine; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30, molecular weight distribution not indicated) by gavage daily on 6 days a week for the first 5 years, whereas for the remaining 2.5 years, carrageenan was incorporated into diet and given to monkeys. In this study, there was also no evidence for the uptake of fibrillar, metachromic, carrageenan-like material in the cells of the reticular-endothelial system in the liver, lymph nodes, caecum, colon or anorectal junction.

In the study of Pittman et al. (1976), rhesus monkeys of both sexes (n = 2) were given carrageenan from *C. crispus* (number-average molecular weight: 185 kDa, ratio ι:κ:λ type 0:70:30, Marine Colloids Inc.), daily in doses of 50, 200 or 500 mg/kg bw per day by gavage. Overnight collections of faeces were obtained after 11 months. Overnight collections of urine were obtained from two of the monkeys on the high dose after 15 months. Two female rhesus monkeys received a single dose of 1 g (about 200 mg/kg bw) of low molecular weight carrageenan from *E. spinosum* (number-average molecular weight: 9.9 kDa, ratio ι; κ; λ type 100:0:0, Glaxo Lab.) by gavage and complete first-day and second-day urine collections were made. In animals receiving carrageenan of number-average molecular weight: 185 kDa no or only traces of carrageenan were excreted in urine. By contrast, when the monkeys were given low molecular weight carrageenan (number-average molecular weight: 9.9 kDa), this type of carrageenan was excreted in urine. From these studies, the Panel noted

that by contrast to carrageenan with a number-average molecular weight of 185 kDa, the lower molecular weight carrageenan would be more absorbed and further excreted in urine. The Panel further noted that the degraded carrageenan used in this study was derived from the ι-carrageenan only and not from the native κ/λ-carrageenan to which it was compared, making inconsistencies in this comparative study and in another study using C16 (Abraham et al., 1972).

Overall, the Panel noted the absence of metabolic studies of carrageenan in humans. In several *in vivo* studies in rats, guinea pigs, neonatal minipigs, neonatal pigs and monkeys, faecal excretion of carrageenan was shown to be 98–100% of the ingested amount. These studies included rats with normal gut microflora and rats with their gut microflora replaced by human faecal microflora. Several short-term and long-term studies performed in rats dosed via diet containing different types of carrageenan confirmed the absence of tissue storage of this carrageenan. However, degraded carrageenan like C16 or low molecular weight carrageenan (number-weight molecular weight of 88 kDa or less) have been described to be absorbed and to be present in various tissues, namely the liver, and the urine of animals given degraded carrageenan mainly when given in drinking water but also when administered via the diet. However, the Panel noted that in these studies, degraded C16 type derived from ι-carrageenan was compared to native κ/λ-carrageenan, making the comparison inconsistent. Due to the high percentage of administered carrageenan recovered in faeces, its degradation to lower molecular weight carrageenan should be very limited.

The Panel noted that the conditions (duration, pH, temperature) for potential hydrolysis of carrageenan in the GI tract are less extreme than the 'mild' acidic conditions which resulted in the formation of chemically degraded carrageenan (C16). The theoretical possibility that more limited degradation could occur under conditions representative of the *in vivo* situation remains an uncertainty although this has not been observed to any significant extent in the *in vivo* ADME studies reported.

The Panel noted that the tested carrageenan differed in the molecular weight distribution, and the Panel considered that existing results did not allow to evaluate kinetic differences between the different types of native carrageenan (κ, λ and ι) and their corresponding low molecular weight fractions.

3.5.2. Acute toxicity of carrageenan

For rats, the oral LD_{50} for sodium or calcium carrageenan (interpreted by the Panel as salts of carrageenan isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) was 5,650–6,250 and 5,140–5,660 mg/kg bw, respectively (JECFA, 1974). For mice, the oral LD_{50} for sodium or calcium carrageenan was reported to be 8,730–9,670 and 8,710–9,590 mg/kg bw, respectively (JECFA, 1974). For ι-carrageenan (food-grade), a LD_{50} of > 5,000 mg/kg bw was reported in rats (Weiner, 1991). For calcium carrageenan, an oral LD_{50} value of > 10,000 mg/kg bw was determined in Sprague–Dawley rats (Documentation provided to EFSA n. 35). No data were available on acute oral toxicity of degraded carrageenan and processed Eucheuma seaweed.

3.5.3. Short-term and subchronic toxicity

Short-term toxicity studies with carrageenan and degraded carrageenan

Rats

Groups of six male albino rats (no further information about the strain; initial body weights of 48–58 g) were dosed with 0%, 5%, 10% or 20% κ/λ-carrageenan (interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) (equivalent to 0, 5,00, 11,800 or 23,600 mg/kg bw per day) via the diet for 10 weeks (Nilson and Schaller, 1941). No indication about weight-average molecular weight was given. Although treated rats showed more laxative condition, in general the animals grew well. Rats dosed with 20% required significantly more feed and water per gram of weight gain than the control group, and in this group, the weight gain also was about 30% less compared with controls. Three of six rats in this dose group died within 14 days after initiation (most probably due to a change in the bacterial flora), but the necropsies were essentially negative. Other parameters were not examined.

Rats (3–4 weeks old, strain not specified) were divided into groups of 10 males and 10 females, dosed for 50 days with carrageenan (commercial product Carasty-S, from the South Portland Maine Plant (USA), food ingredient product lot no. 10036; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) via diet at dose levels of 0%, 15% or 25% (equivalent to 0, 18,000 or 30,000 mg/kg bw per day) (Documentation provided to EFSA n. 36). No

indication about weight-average molecular weight was given. Body weights were recorded twice weekly and at final sacrifice on day 50. An interim sacrifice was done on day 31 (5 males and 5 females from each group) and at gross examination special attention was given to the liver. The rate of feed consumption for the test animals was generally less than the controls, resulting in a decreased weight gain especially in the early part of the study. In all dosed animals diarrhoea was noted after 4 days in study, which continued throughout the remaining period of the study, and the symptoms were more pronounced at a dietary level of 25%. Only at this dose level, male and female rats also excreted bloody stools. In addition, animals receiving carrageenan started losing hair from the dorsal mid-line surfaces on the back during days 8–50, and this finding was more severe in females of the 25% group. At interim sacrifice, it was noted that dosed animals appeared smaller compared with controls and also internal organs were correspondingly smaller. This effect was most prominent in males dosed with 25%. The livers of all animals were normal in texture and surface appearance, and no histopathological differences caused by dosing with carrageenan were seen. At terminal sacrifice on day 50, no gross pathological abnormalities were noted. Many of the livers examined had a mottled appearance, but this finding also occurred in controls. A histopathological examination of the livers gave no indication of adverse effects in all dose groups. Detailed statistics were not reported in this study.

Pigs

In a study with pigs aged 61–75 days, groups of 3 male and 3 female Danish Landrace pigs were given 0, 50, 200, or 500 mg/kg bw per day of κ-carrageenan (commercial product Carrageenan Genulacta type K 100 degrees, batch 039240, from Copenhagen Pectin Factory Ltd., Denmark; number-average molecular weight of 200 kD; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) over 83 days (Poulsen, 1973). No compound-related deaths were observed; the behaviour, appearance, feed intake and growth rate remained normal. No significant changes were reported in haematological, clinical chemical or urinary parameters. At necropsy, there were no changes in relative organ weights. Histopathological examination was performed on principal organs and tissues. No ulceration or erosions were seen in the gastrointestinal tract. Three pigs (one in the 200 mg/kg bw per day group and two in the 500 mg/kg bw per day group) showed slight changes in the mucous membrane of the large intestine, including a few focal areas with irregular surface and some shift in cellular infiltration pattern. Bacteriological examination of the microbiota of the jejunum, caecum, colon and ampulla of the rectum from all animals, showed that treatment resulted in changes in intestinal microbiota. The total counts of aerobic bacteria were decreased in the colon and rectum and the number of lactobacilli was reduced in the rectum.

Monkeys

Rhesus monkeys (*M. mulatta*; 3 males and 3 females) were given solutions of 1% carrageenan composed of κ- and λ-carrageenan (commercial product from Marine Colloids, Inc., Rockland; lot n. 282400, number-average molecular weight of 800 kD; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) or of degraded carrageenan (2%, 1% or 0.5%) (commercial product from Glaxo, Paris, Batch L4006; interpreted by the Panel as prepared from ι-carrageenan originally isolated from *E. spinosum* with an approximate ratio of ι:κ:λ type 100:0:0, with a number-average and weight-average molecular weights of 16–19 kDa and 20–30 kDa, respectively) in drinking water over a period of 7–11 weeks, followed by recovery for up to 24 weeks (Abraham, 1972). There was no evidence of storage of carrageenan, in contrast to degraded carrageenan which produced extensive reticuloendothelial retention of material that was still present in Kupffer cells 6 months after administration of degraded carrageenan was stopped. The stored material was PAS positive and metachromatic; it occupied sites corresponding to the presence of acid phosphatase and P-glucuronidase. Ultrastructurally, the hepatocytes appeared normal in all groups. The results demonstrated that degraded carrageenan is taken up by, and selectively stored in lysosomes of the reticuloendothelial system, notably in the liver, providing evidence of absorption of the sulfated polysaccharide. Parenchymal cells of liver, kidney, or other organs did not reveal the presence of stored material. In contrast to degraded carrageenan, carrageenan administration did not give rise to stored material in reticuloendothelial cells nor in hepatocytes.

In a study in male and female rhesus monkeys (*Macaca mulatta*), the effects of κ/λ-carrageenan, (commercial product from Marine Colloids, Inc., Rockland; as Gelcarin HMR, lot n. 282400; approximate number-average molecular weight of 800 kDa; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of κ:λ type 70:30), have been compared with those of a

degraded carrageenan (C16: commercial product from Glaxo, Paris, Batch L4006; interpreted by the Panel as prepared from ι-carrageenan originally isolated from *E. spinosum* with an approximate ratio of ι:κ:λ type 100:0:0), with a number-average molecular weight of about 20 kDa (Benitz et al., 1973). Both types of carrageenan were administered in drinking-water for 7–14 week. Monkeys given 1% carrageenan (providing an intake of about 1,300 mg/kg bw per day) all gained weight and stayed in good condition. After 7 and 11 weeks, two animals were sacrificed and the remaining ones were put back on plain tap-water and allowed to recover for 11 weeks. To these animals, increasing daily doses of carrageenan ranging from 50 to 1,250 mg/kg bw per day were given for up to 12 weeks. Five males and five females received various concentrations of C16 in the drinking-water up to week 14. Six animals received a 2% solution, two animals a 1% solution and the remaining two received a 0.5% solution of C16. The corresponding average daily intakes of C16 were calculated by the authors to be 2,900, 1,400 and 700 mg/kg bw per day. For the native carrageenan (κ-/λ- carrageenan), soft stools were reported with sporadic positive results for faecal occult blood with a more or less similar pattern in the control monkeys. Gross and microscopic examination of the intestinal tract revealed no changes attributable to the administration of κ-carrageenan. Of the two animals sacrificed at 7 and 14 weeks, only distal colon focal capillary hyperaemia and sometimes a slight degree of mucosal oedema was reported. All other parts of the gastro-intestinal tract were microscopically normal.

Monkeys given 0.5% or 1.0% degraded carrageenan (C16) solution gained weight, but in those on 2.0% (an intake of approximately 2.9 g/day) weight-losses were considerable. All monkeys on C16 lost blood frequently from the intestinal tract in a dose-related degree and developed some degree of anaemia. The authors reported that the animals receiving the 1% C16 solution appeared to pass blood in faeces continuously, while the two monkeys on 0.5% C16 had positive occult blood in faeces at lower frequency. Pathological changes seen in the colon ranged from shallow mucosal erosions to ulceration associated with cellular infiltration, granulation tissue in the lamina propria and formation of multiple crypt abscesses. The severity of these effects was dose-dependent, and some reversal was indicated in monkeys given 2% C16 for 14 week and then allowed to recover on tap-water for 20–24 week. At necropsy, the intestinal tract in these animals was reported as grossly and microscopically normal although some of these animals showed in the colonic wall ulcers on which surrounding mucosa was hyperaemic and contained markedly dilated capillaries. There were reports of remnants of small, usually almost obliterated crypts, some of them containing a considerable number of red blood cells. Results from the recovery period after C16 treatment demonstrated that C16 was the responsible agent for the reported effects on the colon. These studies in rhesus monkeys demonstrated a definite difference between the effects of degraded carrageenan (C16) and carrageenan. The authors concluded that 'administration of the degraded ι-carrageenan (C16) with a number-average molecular weight of about 20,000 (Blakemore and Dewar, 1970) led to intestinal blood loss and anaemia. The morphological abnormalities were seen in the colon, including the colonic portion of the ileocolic valve, and resembled various stages of human ulcerative colitis, an aspect referred to below. Minor degrees of pathological change such as oedema, hyperaemia and an increased number of macrophages were also found in the caecum. All the effects seen in this group were dose-dependent'. The high molecular carrageenan appeared to be well tolerated, whereas low-molecular degraded carrageenan (C16) induced adverse effects in the intestines characterised by various degrees of ulceration and granuloma formation. The LOAEL of the low molecular weight, degraded carrageenan was approximately 750 mg/kg bw per day, whereas the NOAEL of carrageenan was 1,300 mg/kg bw per day, the only dose tested.

Subchronic toxicity studies with carrageenan

Rats

In groups of 3–5 male and 5–7 female rats (no further information about the strain) fed 2%, 5%, 10%, 15% or 20% (equivalent to 1,800, 4,500, 9,000, 13,500 and 18,000 mg/kg bw per day) of κ/λ-carrageenan (commercial product Seakem, type 6, Marine Colloids Inc., Rockland, Main; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) over 23–148 days via diet, apart from a decrease in weight gain in rats dosed with ≥ 10% no adverse effects were reported (Hawkins and Yaphe, 1965). No indication about weight-average molecular weight was given. As ≥ 90% of the ingested carrageenan was excreted via faeces, the authors concluded that it had no direct nutritional value.

Three groups (15 males and 15 females each) of 30 Sprague–Dawley rats of 4 weeks were exposed to 1 or 5% carrageenan (commercial product *Iridaea* carrageenan from Hercules Inc.,

Copenhagen Pectin Fabrik Ltd., Denmark; interpreted by the Panel as isolated from *Iridaea ciliate* = *Sarcothalia crispate,* mixture of κ and λ) via diet (equivalent to 900 and 4,500 mg/kg bw per day) or with cellulose at a 5% dietary level (13 males and 17 females) for 13 weeks (Abraham et al., 1985). No adverse effects on mortality, food consumption, body weight, haematology, clinical chemistry, urinalysis, faeces, or organ weights were noted in animals exposed to 1% carrageenan. No mortality was observed in animals exposed to 5% carrageenan. No indication about weight-average molecular weight was given. At this dose, one male rat showed diarrhoea in week 8 and 5/30 rats, both males and females (exact numbers not given) showed soft stools during weeks 10–13 indicating gastrointestinal disturbances. An increase in food consumption was observed in males at the highest dose (5%) compared to controls. At the highest dose (5%), in both sexes haemoglobin values were decreased compared with controls, while haematocrit levels and erythrocyte and leukocyte counts were comparable with controls. Decreased levels of Na^+ and K^+ and increased SGPT (serum glutamic pyruvic transaminase) levels were observed in females. The faecal consistency at termination was comparable for rats of all groups and no occult blood was detected. There were also no changes in organ weights between treated and control animals. Histopathological examination revealed no changes attributable to the ingestion of carrageenan for 13 weeks. The Panel considered 4,500 mg/kg bw per day, the highest dose tested as the NOAEL in this study.

Three types of carrageenan (differing in viscosity, cation content, and ratios of κ to λ) were tested on Sprague–Dawley rats: (1) TYPE S-70:30 κ:λ ratio, high viscosity (270 cP) and high sodium (6%), (2) TYPE C-80:20 κ:λ, viscosity of 190 cps and high potassium (6%) and (3) TYPE AX-90:10 κ:λ, low viscosity (26 cP) and high calcium (6%) (Abraham et al., 1985) (for the three types, commercial products *Carastay* carrageenan, from Stauffer Chemical Company; sources unknown; interpreted by the Panel as an approximate ratio of ι:κ:λ type 0:70:30; 0:80:20 and 0:90:10). No indication about weight-average molecular weight was given. Eight groups of 50 animals (25 males and 25 females) received the following treatment via their diets for 13 weeks: group 1 was given ground rodent diet and served as untreated control, group 2 was given 5% cellulose and served as a control, group 3 was given 1% (equivalent to 900 mg/kg bw per day) Type S carrageenan, group 4 was given 5% (equivalent to 4,500 mg/kg bw per day) Type S carrageenan, group 5 was given 1% (equivalent to 900 mg/kg bw per day) Type C carrageenan, group 6 was given 5% (equivalent to 4,500 mg/kg bw per day) Type C carrageenan, group 7 was given 1% (about 900 mg/kg bw per day) Type AX carrageenan, and group 8 was given 5% (about 4,500 mg/kg bw per day) Type AX carrageenan. The remaining 10 males and 10 females of group 9 were killed before the start of the study for the determination of baseline haematology and blood chemistry. All parameters classically recommended in OECD 408 were measured in this study. In total, 8 out of 400 rats from groups 2, 3, 4, 7 and 8 died during the study and the cause of death (pneumonia, cage trauma, technician error) was incidental in each case and not related to treatment with carrageenan. There were no adverse effects on body weights, and food consumption was comparable within all groups. Haematological and clinical chemistry parameters gave no significant differences among the nine groups of rats and there was no increase in faecal occult blood. At necropsy, there were no gross changes caused by the treatment and both qualitative and quantitative analyses of liver tissues gave no indication for tissue residues of carrageenan (Abraham et al., 1985). The Panel considered the NOAEL of this study to be 4,500 mg/kg bw per day for all types of carrageenan tested, the highest doses tested.

A 90-day study was performed in compliance to GLP in Fischer 344 rats (groups of 20 rats per sex) exposed via diet at 2 doses of κ-carrageenan (commercial product lot no. 6371-03; interpreted by the Panel as isolated from *E. cottonii* with an approximate ratio of ι:κ:λ type 0:100:0) with an average molecular weight range of 196–257 kDa (not specified if number- or weight-average molecular weight) and low molecular weight tail of 1.9% and 12% < 50 kDa (mean 7%), respectively (Weiner et al., 2007; Weiner, 2014). Animals were exposed to 25,000 and 50,000 mg/kg diet (equal to 1,656 and 3,394 mg/kg bw per day for males and 1,872 and 3,867 mg/kg bw per day for females). Apart from very limited clinical signs (soft faeces, more pronounced in high dose rats), no treatment-related effects were reported on body weights, urinalysis, haematology or clinical chemistry parameters, or on organ weights or ophthalmic, macroscopic or microscopic findings. In addition, the GI tract seemed normal as reported in the detailed histopathological evaluation. According to the authors, the NOAEL was equal to 3,394–3,867 mg/kg bw per day, the highest dose tested in males and females, respectively. The Panel agreed with this NOAEL. The Panel noted that in this study the test material was well characterised and almost complied with the EU specification for E 407.

Subchronic toxicity studies with processed Eucheuma seaweed

The subchronic toxicity of processed Eucheuma seaweed was studied in rats and was compared with effects of carrageenan (Documentation provided to EFSA n. 38). Processed Eucheuma seaweed (interpreted by the Panel as isolated from *E. cottonii* (Ec) with an approximate ratio of ι:κ:λ type 0:100:0 or from *E. spinosum* (Es) with an approximate ratio of ι:κ:λ type 100:0:0) was fed to groups of 20 male and 20 female Sprague–Dawley rats per group (5 weeks old at initiation) at dose levels of 0, 0.5% (equal to 415 and 493 mg processed Eucheuma seaweed -Ec/kg bw per day or 424 mg and 437 processed Eucheuma seaweed-Es/kg bw per day for males and females, respectively), 1.5% (equal to 1,268 and 1,465 mg processed Eucheuma seaweed-Ec/kg bw per day or 1,304 mg and 1,471 mg processed Eucheuma seaweed-Es/kg bw per day for males and females, respectively), or 5% (equal to 4,342 and 4,992 mg processed Eucheuma seaweed-Ec/kg bw per day or 4,484 mg and 5,068 processed Eucheuma seaweed-Es/kg bw per day for males and females, respectively) in the diet for 91–94 days. No indication about the weight-average molecular weight was given. Control groups of 30 male and 30 female Sprague–Dawley rats were given the basal diet for the same period. In additional reference groups of 10 male and 10 female Sprague–Dawley rats, the animals were fed diets for the same treatment period containing 5% carrageenan derived also from *E. cottonii* (carrageenan-Ec; equal to 4,363 and 5,039 mg/kg bw per day for males and females, respectively) or *E. spinosum* (carrageenan-Es; equal to 4,455 and 5,048 mg/kg bw per day for males and females, respectively). In further five groups, the reversibility of effects was studied after a post-exposure observation period; these additional groups of 10 male and 10 female rats were fed the control diet or 5% processed Eucheuma seaweed (processed Eucheuma seaweed-Ec or processed Eucheuma seaweed-Es) or 5% carrageenan (carrageenan-Ec or carrageenan-Es) diets for 90 days and then basal diet for 28 days.

No treatment-related clinical signs were detected in any group as well as no ophthalmological abnormalities. No effects on the body weight of male rats were measured in the low- and mid-dose groups. A slight but statistically significant reduction in the body weight was reported in male rats receiving 5% processed Eucheuma seaweed-Ec (approximately 4% lower than the control; which was also present after the recovery period) or 5% processed Eucheuma seaweed-Es (approximately 5% lower than the control; no effects in the recovery period). The reference group exposed to 5% carrageenan-Ec showed no reduced body weight in males but not the group exposed to 5% carrageenan-Es did (maximum 10% lower than the control, also after the recovery period). No effects were detected in any group of female rats fed processed Eucheuma seaweed-Ec or processed Eucheuma seaweed-Es as well as females receiving 5% carrageenan-Ec. A slight but significant reduction of body weight was found in females exposed to 5% carrageenan-Es (4–6% lower than the controls, also in the reversal phase). The food consumption of treated male rats showed no consistent changes in groups fed with processed Eucheuma seaweed-Ec or carrageenan-Ec. In the functional testing battery at week 12, no dose-related changes were recorded in any treatment group. The urine analysis revealed a significantly higher refractive index and a lower pH value in male rats fed 5% carrageenan-Es. In female rats, the urine volume was significantly increased after exposure to 5% processed Eucheuma seaweed -Es. The same effect plus a lowered refractive index were seen after 5% carrageenan-Ec. Significantly lower levels of ketones were excreted from females fed 5% carrageenan-Ec and 5% carrageenan-ES. After the recovery period, significantly lower urine pH was seen in the male 5% processed Eucheuma seaweed–Ec-treated group and lower proportions of urates in the urine of females from the 5% processed Eucheuma seaweed-Ec and 5% CP-Ec treatment groups. Other effects in urinalysis were not dose-dependent. In the JECFA evaluation (1999), it was concluded that the urinalysis provided no evidence of any toxicologically relevant effects. The Panel agreed with this conclusion. Statistically significant alterations in haematological parameters were reported (e.g. reduced erythrocyte or monocyte counts in males exposed to 5% processed Eucheuma seaweed-Ec; reduced eosinophil counts in males after 5% processed Eucheuma seaweed -ES; reduced haemoglobin, haematocrit, erythrocyte and white blood cell counts in females fed 5% processed Eucheuma seaweed-Ec). Historical control data of this laboratory were not presented. However, the Panel considered the toxicological relevance of these findings questionable since the differences to the concurrent control were marginal, as was also the case with carrageenan-Ec and carrageenan-Es. Clinical chemistry data revealed no effects in any treatment group. In this subchronic study with processed Eucheuma seaweed and carrageenan, no treatment-related clinical signs were detected and only minor effects on body weight or on parameters measured in the functional testing battery, urinalysis, haematology and clinical chemistry were seen. These effects as well as the increase in the

relative organ weights of the full and empty caecum, which was regarded by the Panel as the consequence of accumulation of poorly absorbed material in the intestinal tract, were considered to be without toxicological relevance. No treatment-related histopathological changes were found. Comparing the effects of processed Eucheuma seaweed with the carrageenan of the same seaweed species there was no evidence for any difference in toxicological properties after repeated oral administration. Both preparations of processed Eucheuma seaweed and carrageenan altered the nutrition level available to rats at a concentration of 5% in the diet resulting in increased food intake due to the low nutrient value.

Conclusions for short-term and subchronic studies with carrageenan, degraded carrageenan and processed Eucheuma seaweed

Overall, short-term and subchronic administration of carrageenan was well tolerated and induced no adverse effects in the GI tract in rats, guinea pigs, pigs and monkeys.

Monkeys given degraded carrageenan showed histopathological lesions in the colon which varied from slight mucosal erosions at the low dose (750 mg/kg bw per day) to ulceration associated with inflammatory infiltration of the lamina propria at the high dose (2,900 mg/kg bw per day). The Panel noted a LOAEL of 750 mg degraded ι-carrageenan (C16)/kg bw per day.

The NOAEL for processed Eucheuma seaweed was approximately 4,500 mg/kg bw per day, the highest dose tested.

3.5.4. Genotoxicity

Carrageenan

In vitro

Calcium carrageenan (commercial product FDA 71-5. 977052-14-4; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30, (number- or weight-average molecular weight not specified) was assessed for its mutagenicity in a bacterial mutation assay with *Salmonella* Typhimurium tester strains TA1535, TA1537 and TA1538 and for mitotic recombination in *Saccharomyces cerevisiae* (strain D-4) both with and without S9 metabolic activation (Documentation provided to EFSA n. 39). At the tested concentrations of 0.55%, 1.1% or 2.2% for *S.* Typhimurium and 0.24%, 0.49% or 0.98% for *S. cerevisiae* D4, negative results were obtained. However, the Panel noted that the study bears significant shortcomings in the experimental design which include the use of a limited number of *S.* Typhimurium strains, and that mitotic recombination in *S. cerevisiae* (strain D-4) is considered obsolete and currently not employed for the evaluation of genotoxicity of dose levels employed. On this basis, the Panel considered this study of limited reliability.

Semi-refined carrageenan 'SRC-EC' or 'SRC-ES' (commercial product from Seaweed Industry Association of the Philippines; interpreted by the Panel as isolated from *E. cottonii* with an approximate ratio of ι:κ:λ type 0:100:0, and from *E. spinosum* with an approximate ratio of ι:κ:λ type 100:0:0, number- or weight-average molecular weight not specified, respectively) were assayed for their mutagenicity in a bacterial reverse mutation assay according to the relevant OECD TG Guideline 471 and in compliance with GLP regulations. At the tested concentrations of 0.001–10 mg/plate, both with and without metabolic activation, there was no indication for mutagenicity in the tester strains TA1535, TA1537, TA98, TA100 and TA102 (Documentation provided to EFSA n. 40).

Food-grade carrageenan (commercial product purchased on market place as food grade and analysed by the South Korean advanced Food Research Institute according to Korea Food Additive Code; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30, number- or weight-average molecular weight not specified) was tested in a bacterial reverse mutation assay. At the tested concentrations of 156.3–5,000 μg/plate, both with and without metabolic activation, there was no indication for mutagenicity in the tester strains of *S.* Typhimurium TA1535, TA1537, TA98, TA100 and in *Escherichia coli* WP2uvrA (Chung et al., 2009). The Panel noted that the study meets the requirements of the relevant OECD TG Guideline no. 471.

Sodium carrageenan (interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30, number- or weight-average molecular weight not specified) was assessed for its capability to induce chromosomal aberrations in anaphase in human embryonic lung cells (WI38) at dose levels of 8, 80 and 800 μg/mL in the absence of S9 metabolic activation only, and no cytogenetic effects were reported (Documentation provided to EFSA n. 41). In contrast, calcium carrageenan (not specified further) assayed in a different laboratory (Documentation provided to EFSA n. 42) produced

severe adverse effects at chromosomal level (mainly multipolar spindle mitosis) in the anaphase chromosomes of human embryonic lung cells (WI38) at 10, 100 and 1,000 μg/mL, at the 24 h sampling time. However, at the two higher dose levels, the authors noted a marked discrepancy in the values of individual scoring, indicating that a technical error could have occurred in which two replicate cultures from the intermediate and highest dose levels were reversed. According to the authors, if this was the case, calcium carrageenan would have then yield significant levels of aberrant cells only at one dose level (high dose level), while having no effect at the other two levels. This prompted the authors to repeat the study using the same dose level but inexplicably using an extended sampling time of 42 h. Under these experimental conditions, an extensive chromosome damage irrespective of the tested dose was observed. However, the Panel noted that, in addition to the possible technical error occurred, the positive outcome for chromosomal aberrations at both treatment times could be related to indirect cytotoxic effects not clearly evaluated in this study and therefore the results obtained are questionable.

The Panel also noted that the test on induction of chromosomal aberrations in anaphase did not receive further validation and is currently not employed in genetic toxicology testing. The Panel also noted that the unique positive results in this assay have been reported by this laboratory with another high molecular weight polysaccharide (EFSA ANS Panel, 2017), and overall considered the results from this study not relevant for the risk assessment.

Food-grade carrageenan (commercial product purchased on market place as food grade and analysed by the South Korean Food Research Institute according to Korea Food Additive Code; the origin cannot be interpreted by the Panel, number- or weight-average molecular weight not specified) was assessed for the induction of chromosomal aberrations in Chinese hamster lung fibroblast (CHL), and for induction of micronuclei and DNA breakage by means of the alkaline comet assay in the L5178Y mouse lymphoma cells (Chung et al., 2009). For the induction of chromosomal aberrations, the assay consisted of short-term (6 h) both in the absence and presence of S9 metabolic activation and continuous (24 h) treatments in the absence of S9 metabolic activation at dose levels of 150, 300 and 600 μg/mL. Approximately 22 h after the beginning of the treatment, colcemid was added to each culture at a final concentration of 0.25 μg/mL. 200 metaphases (100 metaphases from each duplicate culture) were selected and analysed for each treatment group and the results were expressed as mean aberrant metaphases excluding gaps per 100 metaphases. At the tested concentrations of 150–600 μg/mL, there was no indication of a clastogenic effect. The Panel noted that the study is compliant with the OECD TG Guideline no 473.

For the induction of micronuclei, L5178Y mouse lymphoma cells were treated with carrageenan at concentrations of 0, 50, 100 or 200 μg/mL for 3 h, both, with and without S9 mix and harvested after a 21-h recovery period, or treated at concentrations of 0, 50, 100 or 200 μg/mL for 24 h without S9 mix and harvested immediately. No duplicate cultures were used and micronuclei were scored in 2,000 cells per culture. Slight but not significant increases in micronucleated cells compared to the concurrent vehicle control were observed at all dose levels and treatment performed. The observed increases were considered by the authors to be of no biological relevance (Chung et al., 2009). The Panel agrees with this conclusion and noted that the study is compliant with the OECD TG no. 487, with the exception that the highest dose level selected was not sufficiently high due to the absence of cytotoxicity and did not show any precipitation of test compound in culture medium as observed in the chromosomal aberration assay. In addition, no historical control range values were reported.

For the comet assay, L5178Y mouse lymphoma cells were similarly treated with carrageenan at concentrations of 0, 50, 100 or 200 μg/mL for 3 h, both, with and without S9 metabolic activation and harvested after a 21-h recovery period, or treated at concentrations of 0, 50, 100 or 200 μg/mL for 24 h without S9 metabolic activation and harvested immediately. Duplicate cultures were used and comets were examined from 100 randomly selected nuclei (50 nuclei per culture) using a fluorescence microscope. Images of selected nuclei were analysed using image-analysis software and tail intensity (% of tail DNA) and Olive tail moment were used to measure the damage of DNA damage. Slight but statistically significant increases in % tail DNA and Olive tail moment compared to the concurrent vehicle control were sporadically found. The increases were not dose-related and the observed values were similar to those commonly observed in the untreated controls for this cell line (Chung et al., 2009). However, the Panel noted some shortcomings which include the absence of historical control range values, a limited number of cells scored, a limitation in the selection of the highest dose level which did not cause adequate cytotoxicity, and no precipitation was observed in culture medium as it was in the chromosome aberration assay.

The Panel considered that carrageenan proved to be negative in all end-points tested in this study (Chung et al., 2009).

In vivo

Sodium carrageenan (interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30, number- or weight-average molecular weight not specified) was assessed for its genotoxic properties in the host-mediated assay *in vivo* in mice, in the rat bone marrow chromosomal aberration assay, and in the rat dominant lethal assay. In all assays performed, the treatment regime used consisted of three dose levels 30, 2,500 and 5,000 mg/kg bw administered acutely by oral gavage, as single dose or subacutely using the same dosages as those in the acute study, each day for 5 consecutive days, 24 h apart (Documentation provided to EFSA n. 41).

In the host-mediated assay, 6–10 mice were allocated to each of the five groups for acute treatment and the four groups for subacute treatments. Three dose levels as described above and negative controls for both acute and subacute treatments were employed. Positive control groups were also included in the acute treatment. The indicator organisms used in this study were two histidine auxotroph *S.* Typhimurium strains (his G-46, TA1530) for induction of reverse mutation and a diploid strain (D3) of *S. cerevisiae* for mitotic recombination. In the acute treatment, all animals, immediately after treatment received 2 mL of indicator organism by intraperitoneal injection containing 3×10^8 cells for *S.* Typhimurium strains and 5×10^8 cells for *S. cerevisiae*. Three hours later animals were sacrificed, indicator organisms removed from peritoneal cavity and appropriately plated for scoring of mutant colonies. Results obtained indicated that tester strains *S.* Typhimurium TA1530, G-46 and *S. cerevisiae* D3 did not show any increase in revertants and mitotic recombination, respectively, both in the acute and subacute treatments. The Panel noted that this assay did not receive further validation and is not currently employed for genotoxicity testing.

In the rat bone marrow chromosomal aberration assay, 59 animals in the acute treatment and 18 in the subacute treatment were employed. In the acute studies, animals were sacrificed 6, 24 and 48 h after dosing and in the subacute treatment 6 h after the last dose. Bone marrow erythrocytes were used to prepare cytogenetic slides for chromosome analyses. Fifty metaphase spread for animal were scored for chromosomal aberration analyses. Results obtained show that the frequency of chromosomal damage observed in the sodium carrageenan animal treated groups was within the range of values observed for the concurrent vehicle control groups (0–6%) both in the subacute and acute treatments.

In the rat dominant lethal assay, a total of 10 male random-bread rats were allocated to each of the five groups, (e.g. three dose levels as described above and positive and negative control) for both acute and subacute treatments. Following treatment, the males were sequentially mated to 2 untreated virgin females per 5 days a week for 8 weeks. At the end of 5 days, females were removed from the males and housed separately until sacrifice. Females were sacrificed at 14 days after separation from males and at necropsy the uterus were analysed for early deaths, late fetal deaths and total implantations. Results obtained showed clear negative results.

Similarly, for calcium carrageenan (interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30, number- or weight-average molecular weight not specified) the genotoxic potential was evaluated in the host-mediated assay *in vivo* in mice, in the rat bone marrow chromosomal aberration assay and in the rat dominant lethal assay. In all assays performed, the treatment regime used consisted of three dose levels 30, 2,500 and 5,000 mg/kg bw administered acutely by oral gavage, as single dose or sub acutely using the same dosages as those in the acute study, each day for 5 consecutive days, 24 h apart (Documentation provided to EFSA n. 35).

In the host-mediated assay, a number of 6–10 mice were allocated to each of the five groups for acute treatment and the four groups for subacute treatments. Three dose levels as described above and negative controls for both acute and subacute treatments were employed. Positive control groups were also included in the acute treatment. The indicator organisms used in this study were two histidine auxotroph *S.* Typhimurium strains (his G-46, TA1530) for induction of reverse mutation and a diploid strain (D3) of *S. cerevisiae* for mitotic recombination. In the acute treatment, all animals, immediately after treatment received 2 mL of indicator organism by intraperitoneal injection containing 3×10^8 cells for *S.* Typhimurium strains and 5×10^8 cells for *S. cerevisiae*. Three hours later animals were sacrificed, indicator organisms removed from peritoneal cavity and appropriately plated for scoring of mutant colonies. Results obtained indicated that tester strains *S.* Typhimurium TA1530, G-46 and *S. cerevisiae* D3 did not show any increase in revertants and mitotic recombination, respectively,

both in the acute and subacute treatments. The Panel noted that this assay did not receive further validation and is not currently employed for genotoxicity testing.

In the rat bone marrow chromosomal aberration assay, a total of 59 animals in the acute treatment and 18 in the subacute treatment were employed. In the acute studies, animals were sacrificed 6, 24 and 48 h after dosing and in the subacute treatment 6 h after the last dose. Bone marrow erythrocytes were used to prepare cytogenetic slides for chromosome analyses. Fifty metaphase spread for animal were scored for chromosomal aberration analyses Results obtained indicate that calcium carrageenan did not induce statistically significant increases of chromosomal aberrations at any dose level and sampling time employed both in the subacute and acute treatments.

In the rat dominant lethal assay, a total of 10 male random-bread rats were allocated to each of the five groups, (e.g. three dose levels as described above and positive and negative control) for both acute and subacute treatments. Following treatment, the males were sequentially mated to two untreated virgin females per 5 days a week for 8 weeks. After 5 days, females were separated from males and housed separately until sacrifice. Females were sacrificed 14 days after separation from males, and at necropsy the uterus were analysed for early deaths, late fetal deaths and total implantations. Results obtained showed clear negative results. The results obtained in these *in vivo* assays indicated that both sodium and calcium carrageenan were not systemically genotoxic. However, the Panel noted that these results could be anticipated due to the negligible absorption of both sodium and calcium carrageenan.

In summary, carrageenan (native, food-grade, sodium and calcium salts) has been tested in several *in vitro* and *in vivo* genotoxicity tests. Negative results from *in vitro* studies of high relevance and OECD guideline compliance were observed in bacterial gene mutation assays with semi-refined carrageenan from *E. cottonii* or *E. spinosum* sources (Documentation provided to EFSA n. 40) and with food-grade carrageenan (Chung et al., 2009).

Similarly, in mammalian cells, food-grade carrageenan proved to be negative for induction of chromosomal aberrations in CHL and micronuclei in L5178Y mouse lymphoma (Chung et al., 2009). The Panel noted that this combination of tests fulfilled the basic requirements to cover the three genetic endpoints (e.g. point mutation, structural and numerical chromosomal aberrations) required for the assessment of the genotoxicity of a test compound (EFSA Scientific Committee Guidance, 2011).

Negative results from limited studies were also observed *in vitro* in a bacterial gene mutation assay for calcium carrageenan (Documentation provided to EFSA n. 39), in a chromosomal aberration assay in anaphase in human embryonic lung (WI38) cells (Documentation provided to EFSA n. 41) for sodium carrageenan and *in vivo* in a host mediated assay in mice and in a chromosomal aberration and a dominant lethal assays in rats (Documentation provided to EFSA n. 41; Documentation provided to EFSA n. 35) for both sodium and calcium carrageenan.

Overall, carrageenan did not show genotoxic activities from a robust data set.

Processed Eucheuma seaweed

In vitro

Semi-refined carrageenan (commercial product from Seaweed Industry Association of the Philippines; interpreted by the Panel as isolated from *E. cottonii* with an approximate ratio of ι:κ:λ type 0:100:0, and from *E. spinosum* with an approximate ratio of ι:κ:λ type 100:0:0, number- or weight-average molecular weight not specified) from two sources (e.g. *E. cottonii* or *E. spinosum*), respectively, were assayed for their mutagenicity in a bacterial reverse mutation assay according to the relevant OECD TG Guideline 471 and in compliance with GLP. At the tested concentrations of 0.001–10 mg/plate, both with and without metabolic activation, there was no indication for mutagenicity in the tester strains TA1535, TA1537, TA98, TA100 and TA102 (Documentation provided to EFSA n. 40).

In the study by Sylianco et al. (1993), processed Eucheuma seaweed (identified as PNG carrageenan, without no further details) was assayed for its mutagenicity in a bacterial reverse mutation assay at concentrations of 25, 50 or 100 mg/mL in the absence of S9 metabolic activation only. The assay was performed using *S.* Typhimurium TA100 tester strain. The Panel noted that the study has severe limitations which included the absence of treatment in the presence of S9 metabolism and the use of the single *S.* Typhimurium TA100 tester strain. Concentration were only reported in limited tables as 25, 50 or 100 mg/mL and therefore it was not known which concentration was administered to the plates, the number of plates was not indicated, the number of colonies in the positive control was not reported. No data were given on cytotoxicity. Overall, the Panel concluded that the study was not suitable to be considered for risk assessment.

In the rec-assay using *Bacillus subtilis* rec$^-$ and *B. subtilis* rec$^+$, no DNA damaging activity was found at concentrations of 25, 50 or 100 mg/mL of processed Eucheuma seaweed (identified as PNG carrageenan, without no further details) (Sylianco et al., 1993). The Panel noted that the study has severe limitations which include the absence of treatment in the presence of S9 metabolism, concentration were only reported in limited tables as 25, 50 or 100 mg/mL and therefore it is not known which concentration was administered to the plates and the *B. subtilis* rec$^-$ and rec$^+$ employed were not reported. Overall, the Panel concluded that the study was not suitable to be considered for risk assessment.

In vivo

In the mouse bone marrow micronucleus assay (Sylianco et al., 1993), Swiss Webster mice (sex and number of animals not stated) were administered by oral gavage twice with processed Eucheuma seaweed (identified as PNG carrageenan, without further details) at dose levels of 625, 1,250 or 2,500 mg/kg bw, 24 h apart. Vehicle (water) and positive control (dimethylnitrosamine) treatment groups were also included. Six hours after the last treatment, the mice were sacrificed and bone marrow was processed for analysis of micronucleated polychromatic erythrocytes (no data about cytotoxicity clinical signs or number of cells analysed). In all treatment groups, the number of micronucleated cells was not increased apart from the positive (no data about the dose). The Panel noted that the results of this study are of limited relevance due to the absence of information on the number of polychromatic erythrocytes scored for the induction of micronuclei and cytotoxicity not evaluated. In addition, the Panel noted that PNG carrageenan is poorly absorbed.

No mutagenic properties were reported in the host-mediated assay (Sylianco et al., 1993). Swiss Webster mice (sex and number of animals not stated) received via gavage 0 (vehicle control, water), 625, 1,250 or 2,500 mg/kg bw processed Eucheuma seaweed (number- or weight-average molecular weight not specified) and the indicator organisms (*S.* Typhimurium strain G46) were injected into the peritoneum (no further details). The positive control benzo(a)pyrene resulted in a fivefold increase of the mutation frequency. The Panel noted that the study was poorly reported and that it has not received further validation and it is presently considered obsolete.

In summary, processed Eucheuma seaweed derived from either *E. cottonii* or *E. spinosum* was not mutagenic in a OECD guideline compliant bacterial reverse mutation assay (Documentation provided to EFSA n. 40). Additional *in vitro* studies (bacterial reverse mutation test and rec assay) resulted also in no genotoxic activity (Sylianco et al., 1993). However, these studies suffered from methodological weaknesses and limited information. Similarly *in vivo*, in studies with limited reliability, no clastogenic or aneugenic activity and no mutagenic activities were found in a mouse bone marrow micronucleus assay and in an host mediated assay, respectively (Sylianco et al., 1993).

Overall, despite the limitation of the available data the Panel considered processed Euchema seaweed to be of no genotoxic concern.

Degraded carrageenan

In vitro

Degraded carrageenan (not further specified) was tested for its mutagenicity in a bacterial reverse mutation assay. At the tested concentrations of 100–5,000 μg/plate, both with and without metabolic activation, there was no indication for mutagenicity in the tester strains of *S.* Typhimurium TA98 and TA100 (Wakabayashi, 1981; article in Japanese and summary in English). The Panel considered the negative results observed as reliable with limitations since an incomplete set of *S.* Typhimurium tester strains (only two strains) was employed.

In the study by Mori et al. (1985), degraded carrageenan, with a sulfate content of about 30% and an average molecular weight of 20–40 kDa (number- or weight-average molecular weight not specified), obtained from Glaxo Laboratories (Paris, France, not further characterised), was assayed for induction of gene mutation in *S.* Typhimurium strains TA98 and TA100, both in the absence and presence of S9 metabolic activation at dose levels of 8.900 and 26.700 μg/plate, much higher than the upper limit indicated in the relevant OECD Guideline 471. The results obtained did not show any increase in revertant numbers compared to the concurrent vehicle control. The Panel considered the negative results observed as reliable with limitations since an incomplete set of *S*. Typhimurium tester strains (only two strains) was employed.

In the study by (Wakabayashi, 1981; article in Japanese and summary in English), degraded carrageenan (not further specified) was tested for induction of point mutation in V79 Chinese hamster

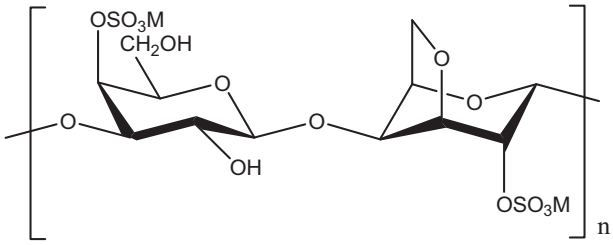
cells at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus, only in the absence of S9 metabolism, at dose levels of 10, 100 and 1,000 μg/mL and negative results were obtained. The Panel noted that the study bears some shortcomings which include the use of a top dose level lower than that commonly recommended despite the absence of any cytotoxicity. In addition, treatments were performed only in the absence of S9 metabolism. On this basis, the Panel considered the results of this study of limited relevance.

In the study by Mori et al. (1985) degraded carrageenan, with a sulfate content of about 30% and an average molecular weight of 20–40 kDa (not specified if number- or weight-average molecular weight), obtained from Glaxo Laboratories (Paris, France, not further characterised), was assayed for induction of DNA repair using the *in vitro* unscheduled DNA synthesis (UDS) assay in rat hepatocytes and intestinal mucosal cells from ileum and colon/rectum at dose levels of 10 and 100 μg/mL as limited by the solubility of the test compound. The results obtained indicated that degraded carrageenan did not produce any evidence of UDS in either the hepatocyte or intestinal mucosal cells. The Panel noted that this assay detects only compounds which specifically trigger DNA excision repair following DNA adduction.

In silico

The evaluation of structural alerts for genotoxicity in the basic chemical structure of the monomer component of degraded carrageenan (as displayed in the figure below adapted from Wakabayashi) was performed with the OECD QSAR Tool box (2.6.13) using the following profilers: 'Alerts for *in vitro* mutagenicity (Ames test) by ISS', 'DNA alerts for Ames, MN, and CA by OASIS v.1.3', 'Alerts for DNA binding (by OASIS v.1.3 and OECD)' and 'Alerts for *in vivo* mutagenicity (Micronucleus) by ISS'.

No relevant structural alerts for genotoxicity were identified with the exception of the alert namely 'H-acceptor-path3-H-acceptor' detected by the profiler 'Structure Alerts for the *in vivo* micronucleus assay' The 'H-acceptor-path3-H-acceptor' refers to the possibility of non-covalent binding to DNA or proteins as a result of the presence of two bonded atoms connecting two hydrogen bond acceptors. However, the Panel noted that the positive predictivity of such alerts for *in vivo* genotoxicity was quite low, ranging from 'none' (34%) to 63% depending on the database, with a high incidence of false positives (Benigni et al., 2010, 2012).



M=Na or K

Summary of findings for degraded carrageenan

Degraded carrageenan (not further specified) was not mutagenic in *S.* Typhimurium TA 98 and TA 100 or in Chinese hamster V79 cells (Wakabayashi, 1981). Mori et al. (1985) confirmed the absence of mutagenicity in *S.* Typhimurium TA 98 and TA 100 tester strains and showed that degraded carrageenan did not exhibit genotoxic activity in DNA repair tests employing rat hepatocytes and intestinal mucosal cells. However, despite the limitations of the above mentioned studies, the absence of genotoxic effects was also indicated by the results of *in silico* evaluation.

Conclusion for genotoxicity

Overall, the Panel concluded that carrageenan and processed Eucheuma seaweed did not raise a concern with respect to genotoxicity. The Panel also noted that degraded carrageenan was not of genotoxic concern; this further supported that the food additives E 407 and E 407a, even if they contain some low molecular weight carrageenan, were not of genotoxic concern.

3.5.5. Chronic toxicity and carcinogenicity on carrageenan and degraded carrageenan

Mice

Male and female mice (10 animals/group, strains not specified) were exposed for the lifetime to diets containing carrageenan (Nilson and Wagner, 1959). Carrageenan (commercial product Krim-Ko-gel, high viscosity, now designated Sea Kem, type 5; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) used in the study (performed from 1951 to 1953) was purchased on the open market except for several lots which were supplied by the manufacturer. The first two lots analysed 76% and 75% hydrocolloid, 12% and 15% moisture, 25% and 22% mineral matter by weight, respectively. The hydrocolloid content was based on precipitating a 2% solution of the sample with 2 volumes of 99% isopropanol. No indication about the weight- average molecular weight was given. The arsenic content varied from 0.04 to 10.4 ppm (10.4 mg/kg). Carrageenan was mixed into the diet at 1%, 5%, or 15% levels (equivalent to 1,500, 7,500, 22,500 mg/kg bw per day) at the expense of equal weights of ground yellow corn and at the 25% (equivalent to 37,500 mg/kg bw per day) level for a fixed ration of ground yellow corn, wheat and oats. The animals were weighed once weekly and also food and water intake was determined once a week. No haematological and clinical parameters were measured. The mice were kept until death and at necropsy a gross examination was performed. If possible, organ sections or entire organs of each animal were preserved, sectioned and examined microscopically. No adverse effects on growth, survival, gross and histopathology were reported in any of the dosed animals.

The Panel noted that different lots of carrageenan have been used during the study, that the presence of arsenic at higher level than EU specifications in the substance has been detected. Moreover, the study has been conducted until the death of animals and there are differences in the feed composition for animals exposed to 25%. Consequently, this study cannot be used for the hazard characterisation.

Rats

A similar study has been performed by Nilson and Wagner (1959) with groups of 10 male and female rats. The strain was not specified. Carrageenan (commercial product Krim-Ko-gel, high viscosity, now designated Sea Kem, type 5; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) was mixed into the diet at 1%, 5% or 15% (equivalent to 500, 2,500, or 7,500 mg/kg bw per day) at the expense of equal weights of ground yellow corn, and at 25% (equivalent to 37,500 mg/kg bw per day) for a fixed ration of ground yellow corn, wheat and oats. No indication about the weight-average molecular weight was given. No adverse effects were noted except at the highest dose (25%) which exhibited lesions suggesting hepatic cirrhosis. According to the authors, these observations could indicate some dietary deficiency or insufficiency since the bulk handled was large and faeces often very loose indicating that mechanical loss of nutrient could not be discounted for this effect.

The Panel noted that different lots of carrageenan have been used during the study, and that the presence of arsenic in the test material was at higher level than EU specifications for the substance. Moreover, the study was conducted until the death of animals and there were differences in the feed composition for animals exposed to 25% carrageenan. Consequently, the Panel noted that this study was not in agreement with the current guidelines for a long-term study used for the hazard characterisation. The Panel noted that JECFA used this study in 1974 to allocate an ADI of 75 mg/kg bw per day, based on a NOAEL of 15% equal according to JECFA to 7,500 mg/kg bw per day. In 1978, the SCF endorsed this value and confirmed it until the latest evaluation in 2003.

Nutritional studies were conducted with Sprague–Dawley rats fed diets containing 4% κ/λ-carrageenan (interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) (equivalent to 2,000 mg/kg bw per day) that had been mixed into skimmed milk at a concentration equal to that of the protein and heat sterilised under conditions routinely used in the manufacture of canned liquid milk (Tomarelli et al., 1974). No indication about the weight-average molecular weight of the tested material was given. The authors only refer to a Food and Drug Administration review (FDA, 1972) in which, to ensure against the introduction of degraded carrageenan into the food supply, have amended the existing Regulation by stipulating that the carrageenan have an average molecular weight exceeding 100 kDa, as indicated by a specified test.[26]

[26] FDA, Fed. Regist., 15434 (2 August 1972).

Two experiments of 6 months duration each were conducted to determine the effect of feeding relatively large amounts of processed carrageenan for prolonged periods. In the first experiment, two dietary groups were maintained: a control group fed the basal diet and a carrageenan group fed the basal diet in which equivalent amounts of the skimmed milk powder and cellulose were replaced by the processed milk-carrageenan product. In the second experiment, a third dietary group was included, a group fed the basal diet in which dextrose was substituted for the cellulose. This group served as a control to assess the influence of the relatively large amounts of indigestible carbohydrate presented by the carrageenan or cellulose. The dietary groups of the first experiment consisted of 25 males and 25 females, while in the second there were 12 males and 12 females per group. In the second experiment half of the rats of each dietary and sex group were killed at the fourth month. The rats were weighed twice weekly. At intervals, 6–8 rats from each of the groups were located in metabolism cages during 5–7 days for studying the absorption of nutrients. At necropsy, the organs were examined for gross lesions with particular attention directed to the lower gastrointestinal tract. The authors reported that, 'when fed in a simulated milk powder diet, the processed carrageenan at a dietary level of 4% had no influence (compared to glucose or cellulose) on general health, growth rate, diet energy efficiency, absorption of protein, fat, calcium, blood coagulation, utilization of protein for growth, or utilization of iron. Gross and microscopic examination of the caecum and colon revealed no abnormalities'.

The Panel considered 4% of carrageenan in the diet (equivalent to 2,000 mg/kg bw per day), which did not induce nutritional imbalance, as a NOAEL.

Several carrageenan products (commercial products from Carrageenans Hercules Inc) extracted from *C. crispus* (77.7% κ, 22.3% λ), *G. acicularis* (23.3% κ, 76.7% λ), and *E. spinosum* (100% ι) were tested in rats by oral administration (Documentation provided to EFSA n. 44). No indication about the weight-average molecular weight was given. Alphacel was used as a bulk replacement control diet. Seven groups of 30 Sprague–Dawley rats (15 males, 15 females) with an age of about 4 weeks were dosed via diet with 1 or 5% (equivalent to 500 and 2,500 mg/kg bw per day) of the carrageenan or with 5% Alphacel over 40 weeks. Mortality and morbidity were recorded daily and animal weights were recorded weekly. Haematological tests included haemoglobin, haematocrit, erythrocyte count, and total and differential leukocyte count. Clinical tests consisted of blood glucose, blood urea nitrogen, serum glutamate pyruvate transaminase, Na^+, K^+, Cl^-, and examination of stools from six males and six females of each group was performed after 40 weeks. At termination, a full complement of tissue specimens was obtained from six male and six female rats dosed with 5% of the different carrageenan and these specimens were prepared and evaluated histologically and also liver portions were processed for electron microscopy. None of the dosed rats died during the study and there was no evidence of toxicity. There were also no differences in mean body weights between dosed rats and controls receiving 5% Alphacel, and the treatment had no adverse effects on haematological and clinical chemistry parameters. The faecal consistency at study termination was comparable for rats of all groups, and occult blood was detected less frequently among rats dosed with carrageenan compared with those rats treated with Alphacel. The dosing with the different carrageenan caused no significant changes of absolute liver weights in dosed rats and the histopathological examination gave no indications for alterations in morphology or for storage of metachromatic material in either Kupffer cells or hepatocytes. The Panel considered that the NOAEL of this study was equal to 5% equivalent to 2,500 mg/kg bw per day the highest dose tested, whatever the carrageenan tested.

Carrageenan (commercial product Gelcarin HMR from Marine Colloids, Inc. Rockland, Ma, Lot No 102012; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) was fed to two strains of weanling rats (Sprague–Dawley albino or Osborne-Mendel) at a 5% equivalent to (2,500 mg/kg bw per day) dietary level in two different basal diets (ground Wane Lab Blox [AMC] or FDA diet) for 39 weeks (Documentation provided to EFSA n. 42). Alphacel was used as a bulk replacement control diet. No indication about the weight-average molecular weight was given. Cellulose fibre was prepared at a 5% dietary level in two basal diets and fed to two strains of rats which served as controls. Rats were assigned to 8 groups of 18 males and 18 females and the rats were fed their respective diets for 39 weeks in a multiple-cross design: group l – Osborne-Mendel rats fed 5% cellulose in FDA diet, group 2 – Osborne-Mendel rats fed 5% carrageenan in FDA diet, group 3 – Osborne-Mendel rats fed 5% cellulose in AMC diet; group 4 – Osborne-Mendel rats fed 5% carrageenan in AMC diet; group 5 – Sprague–Dawley rats fed 5% cellulose in FDA diet; group 6 – Sprague–Dawley rats fed 5% carrageenan in FDA diet; group 7 –Sprague–Dawley rats fed 5% cellulose in AMC diet and group 8 – Sprague–Dawley rats fed 5% carrageenan in AMC diet. Mortality

and morbidity were recorded twice daily, and body weight, physical appearance, food and water consumption, and stool consistency (soft, firm or semifluid) were recorded at intervals. The presence of faecal occult blood was also monitored. The animals were killed and grossly examined at 30 days (3 males and 3 females per group) and 90 days (5 males and five females per group), while the surviving 160 rats were killed after 38 weeks and examined grossly. In addition, final body weights and absolute and relative liver weights were noted. Portions of the liver from all rats killed after 39 weeks were prepared for histochemical and ultrastructural determination of carrageenan storage and also a microscopic examination was undertaken. The treatment caused no mortality and all rats were in good health. Initially the body weights of Osborne-Mendel rats were lower compared with Sprague–Dawley rats, however after 39 weeks the Osborne-Mendel rats weighed more than the Sprague–Dawley strain. The dosing of carrageenan in AMC diet caused lower body weights in Sprague–Dawley males (group 8) at 4 weeks and in Osborne-Mendel females (group 4) throughout the experimental period. Soft faeces were noted in rats of all carrageenan groups. Occult blood in the faeces was sporadic in all groups (in control and treated groups) in time of appearance and quantity; occult blood was detected less frequently among rats dosed with carrageenan compared with those rats in the Alphacel group. Only Osborne-Mendel rats fed 5% carrageenan in FDA diet showed significantly increased (not further specified) relative liver weights after 39 weeks. At interim sacrifice on day 90, all rats given cellulose diet were grossly normal. Only two carrageenan fed rats (one male from group 8 and one female from group 2) had gross hepatic changes characterised as lobular flattening with a rough capsular surface. At the histopathological examination, both the 5% cellulose and the 5% carrageenan diets caused hepatocellular pericentral atrophy, hepatocellular necrosis and apoptosis. In some instances, the mitotic activity either was moderate or increased. There was a moderate degree of sinusoidal hyperaemia and varying degrees of hepatocyte vacuolisation. Bile duct proliferation was observed only in the livers of rats given carrageenan. For all of these findings, no statistics were given (Documentation provided to EFSA n. 42). The Panel considered that the 5% in the diet equivalent to 2,500 mg/kg bw per day, the highest dose tested, was the NOAEL.

Groups of 15 male and female Sprague–Dawley rats were dosed with two extracts of carrageenan (one with molecular weight estimate of 244 kD, interpreted by the Panel as isolated from *H. musciformis* similar to κ; or the second one with molecular weight estimate of 252 kD, interpreted by the Panel as isolated from *S. crispata* (synonym: *Iridaea crispata*) lying between λ and κ properties) at a concentration of 0%, 1% or 5% (equivalent to 0, 500 and 2,500 mg/kg bw per day) in the diet for one year (Documentation provided to EFSA n. 43). No indication about weight-average molecular weight was given. Controls received 5% Alphacel in the diet. In rats dosed with 1% there were no changes in the stools, while female rats dosed with 5% carrageenan from *S. crispata* (*I. crispate)* and males dosed either carrageenan at the 5% concentration had loose stools. Blood was observed sporadically in the stools, but the frequency was lower than in the controls. At the end of the study, body weights of the carrageenan-treated groups were decreased by 20% or 25% in the 1% and 5% carrageenan groups, respectively. In the group dosed with 1% carrageenan from *H. musciformis* the absolute and relative liver weights of males were comparable to the controls, while the absolute and relative liver weights of females were decreased when compared to the controls (absolute: 9.0 vs 15.6 g; relative 3.0 vs 4.0 g). In the group dosed with 5% carrageenan from *H. musciformis*, the absolute liver weights of males and females were decreased compared to the controls (males: 14.9 vs 18.7 g; females 8.4 vs 15.6 g) and the relative liver weight of females was decreased compared to the controls (2.9 vs. 4.0 g). In the group dosed with 1% carrageenan from *S. crispata* (*I. crispate)* the absolute liver weight of males was comparable to the controls but the relative weigh was increased (3.1 vs. 2.7 g). For females, both the absolute and relative liver weights were decreased compared to the controls (absolute: 9.8 vs 15.6 g; relative 3.2 vs 4.0 for the controls). In the group dosed with 5% carrageenan from *S. crispata* (*I. crispata*) the absolute liver weights of the females were lower than in the controls (8.2 vs 15.6 g). For this group the relative liver weights of the males were higher than the controls (3.0 vs 2.7 g) and the relative liver weight of the females was lower compared to the controls (2.9 vs 4.0 g). Some incidental effects were observed at macroscopical and histopathological examination of the liver. The dosing gave no indication for storage of carrageenan-like material in the liver cells of any of the treated rats, and no fibrillar material was seen by electron microscopy. Except for gross changes in the livers, no other adverse effects were observed. The Panel considered that the LOAEL in this study was 1% in the diet (equivalent to 500 mg/kg bw per day) for both carrageenan preparations.

Groups of 30 male and 30 female MRC outbred rats were dosed lifetime with 0%, 0.5%, 2.5% or 5% of carrageenan (commercial product Gelcarin HMR from Marine Colloids, Inc., Rockland, ME, Lot.

No 102012; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30), in the diet (Rustia et al., 1980). Carrageenan (Gelcarin) was reported as having a molecular weight of approximately 800 kDa. No indication about weight-average molecular weight was given. The average daily carrageenan intake was equal to 0, 360, 1,998 and 4,022 mg/kg bw. As untreated controls, 100 females and 100 males were used. The animals were inspected routinely two times a day and food consumption was recorded twice a week for a 2-day period. Animals were allowed to die spontaneously or killed when moribund. Complete autopsies were performed on all experimental and control animals, except for a few that were cannibalised or showed advanced post-mortem deterioration. Histological specimens were taken from all visceral organs and from all tumours and pathologically altered tissues. All segments of the gastrointestinal tract including the oesophagus, glandular steroids and forestomach, small intestine/duodenum, jejunum and ileum, cecum, colon and rectum were examined for lesions. The other tissues were fixed, processed and stained routinely with haematoxylin and eosin. The data including tumour and death rates were statistically analysed. The animals tolerated the carrageenan well at all dose levels and exhibited no clinical signs of excessive toxicity (e.g. significant body weight reduction). Animals occasionally developed a soft stool consistency, particularly in the initial experimental stages, and also diarrhoea was noted in some animals. No ulcerative alterations or neoplastic lesions of the intestinal tract were observed and the number of tumour-bearing animals, tumours per tumour-bearing animal and the overall incidence (i.e. number of tumours in test and control animals) and average latency periods did not differ significantly among test and control groups). According to the Panel, the results of this study, demonstrated no carcinogenic effects of carrageenan in rat. The Panel considered that the NOAEL of this study was 4,022 mg/kg bw per day, the highest dose tested.

Syrian golden hamsters

In a study with Syrian golden hamsters, groups of 30 male and 30 female hamsters were dosed livelong with 0%, 0.5%, 2.5% or 5% of carrageenan (commercial product Gelcarin HMR from Marine Colloids, Inc., Rockland, ME, Lot. No 102012; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) (Rustia et al., 1980). Carrageenan (Gelcarin) was reported as having a molecular weight of approximately 800 kDa. No indication about weight-average molecular weight was given. The average daily carrageenan intake was equal to 0, 370, 2,162 or 3,719 mg/kg bw per day. As untreated controls, 100 females and 100 males were used. The animals were inspected routinely two times a day and food consumption was recorded twice a week for a 2-day period. Animals were allowed to die spontaneously or killed when moribund. Complete autopsies were performed on all experimental and control animals, except for a few that were cannibalised or showed advanced post-mortem deterioration. Histological specimens were taken from all visceral organs and from all tumours and pathologically altered tissues. All segments of the gastrointestinal tract including the oesophagus, glandular steroids and forestomach, small intestine/duodenum, jejunum and ileum, cecum, colon and rectum were examined for lesions. The other tissues were fixed, processed and stained routinely with haematoxylin and eosin. The data including tumour and death rates were statistically analysed. The animals tolerated the carrageenan well at all dose levels and exhibited no clinical signs of excessive toxicity (e.g. significant body weight reduction). Animals given the highest dose level of carrageenan had soft stools during the initial weeks of the experiment. No ulcerative alterations or neoplastic lesions of the intestinal tract were observed and the number of tumour-bearing animals, tumours per tumour-bearing animal and the overall incidence (i.e. number of tumours in test and control animals) and average latency periods did not differ significantly among test and control groups). From the results of this study, carrageenan demonstrated no carcinogenic effects in Syrian golden hamsters. The Panel considered that the NOAEL of this study was equal to 3,719 mg/kg bw per day the highest dose tested.

Monkeys

A long–term toxicity study (7.5 years) has been performed on 40 rhesus monkeys (19 males and 21 females with initial weights of 2–8.22 kg) which were divided into 4 groups with 10 animals each and dosed with 0, 50, 200, 500 mg/kg bw per day κ/λ-carrageenan (commercial product Gelcarin HMR from Marine Colloids, Rockland, Maine; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30) by gavage daily on six days a week for the first 5 years (after about one year 10/19 males became aggressive and were dosed via diet) (Documentation provided to EFSA n. 34). No indication about weight-average molecular weight was given. Thereafter, for the remaining 2.5 years, carrageenan was incorporated into diet and given to monkeys. Observations on

appearance and behaviour were done on 6–7 days/week, while body weights were recorded weekly for the first month, and monthly thereafter and before termination. Clinical chemistry was done twice prior to start and at 2–12 months, in years 4–7 and prior to termination. A hepatic biopsy was performed on selected monkeys after 9 months and in years 2, 3.75, 5 and 6 of the study. Necropsy and gross examination was done in 6 of 10 monkeys which died or were killed (2–3 animals within all groups) for humane reasons during the study and at termination. The organ weights of liver, kidneys, heart, spleen, ovaries, testes, brain, adrenals and pituitary were determined and a microscopic examination of all major relevant organs was done. Histopathological examination (staining with toluidine blue) and electron microscopy was done in portions of the liver, large and small intestine, caecum and lymph nodes from each monkey. No statistical differences in mean survival times were detected in any group as compared to controls and there were also no gross or microscopic changes in the examined tissues. During years 5–7.5, in females dosed with 50 mg/kg bw per day a significant decrease in body weights was seen. Loose stools, chronic intestinal disorders, poor appetite and emaciation were observed in an apparently random distribution. Stool consistency was decreased in a dose-related manner over 7.5 years (the incidence of soft stools as a frequency of total observations was 9.1%, 18.7%, 20.3% and 24.4% in controls, low, mid- and high-dose groups) while positive faecal occult blood findings were increased in a similar fashion; the values obtained ranged from 16.8% in controls to 25.4% in the high-dose group. Faeces of monkeys given carrageenan were found to contain degraded carrageenan. Therefore, the Panel noted that it cannot be excluded that the presence of occult blood in animals is treatment-related. There were no changes in haematology, clinical chemistry values, organ weights, gross pathology, microscopic and ultrastructural morphology. There was also no evidence for the uptake of fibrillar, metachromic, carrageenan-like material in the cells of the reticular-endothelial system in the liver, lymph nodes, caecum, colon or anorectal junction. Three neoplastic changes were noted at termination: one perianal tumour in one control female, a cervical polyp in one female at 50 mg/kg bw per day and a fibroma of the thigh in one male at 200 mg/kg bw per day).

The Panel noted that the chronic toxicity studies were almost exclusively conducted with κ/λ-carrageenan and that no or no adequate description of the molecular weight distribution of the tested carrageenan preparations was given. There was no indication of carcinogenicity related to the treatment.

Initiation–Promotion studies

Several initiation–promotion studies with equivocal data were identified in the scientific literature by the Panel. Overall, the Panel considered that the results of the initiation–promotion studies did not demonstrate consistent modulating effects of carrageenan on the initiation and/or promotion phase of colon carcinogenesis induced in rats by well-known carcinogenic compounds such as azoxymethane (AOM) (Watanabe et al., 1978; Corpet et al., 1997; Taché et al., 2000) and dimethyhydrazine (DMH) (Arakawa et al., 1986; Hagiwara et al., 2001). Watanabe et al. (1978) and Arakawa et al. (1986) used doses up to 15% of the diet and were in excess of those accepted as maximum (5%) for a chronic bioassay (OECD, 1998). Aberrant crypt foci were used as putative preneoplastic (surrogate) markers for the development of colon cancer in the studies of Corpet et al. (1997) and Taché et al. (2000). They found a significant decrease in the number of aberrant crypt foci. Furthermore, no plausible mechanism of action has been proposed that could explain the results observed.

Overall, the Panel considered that there was no concern with respect to the carcinogenicity of carrageenan in several chronic toxicity studies in rats performed with carrageenan (mostly κ/λ-type, molecular weight distribution not adequately described). From the available rats studies, NOAELs up to 7,500 mg/kg bw per day, the highest dose tested, were identified (Nilson and Wagner, 1959). However, in another study in rats given carrageenan preparations with a molecular weight of 244 kDa and 252 kDa, a LOAEL of 1% in the diet (equivalent to 500 mg/kg bw per day) was identified by the Panel (Documentation provided to EFSA n. 43). The Panel noted that the characterisation of the test material in all the chronic studies was limited.

No data were available on chronic oral toxicity and carcinogenicity of processed Eucheuma seaweed. Studies with degraded carrageenan showing carcinogenicity have been described under Section 3.5.8. (Other studies).

3.5.6. Reproductive and developmental toxicity

Reproductive toxicity studies

High molecular calcium carrageenan (κ-λ) (commercial product Gelcarin-HMR from Marine Colloids, Rockland, Maine; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30, no indication of molecular weight distribution) was tested in a combined three-generation reproduction toxicity and developmental study (the latter dealt with in section Developmental Toxicity) with groups of 40 male and 40 female Osborne-Mendel rats (Collins et al., 1977a). The just weaned animals were dosed with diets containing calcium carrageenan at levels of 0, 5,000, 10,000, 25,000 or 50,000 mg/kg diet. The authors calculated daily carrageenan consumption for adult males was 0, 290, 592, 1,555 or 3,212 mg/kg bw per day and for adult females 0, 350, 711, 1,832 or 3,805 mg/kg bw per day. After weaning, all animals were fed carrageenan in their diets for 12 weeks before mating. Animals of both F_{1A} and F_{1B} litters were sexed and weighed at birth and weighed also on days 4, 7, 14 and 21. Checking for abnormalities in all dams and pups was done until weaning at PND 21, when F_{1A} rats were autopsied and discarded. Randomly selected F_{1B} and F_{2B} rats were mated to produce the F_2 and F_3 generations, respectively. F_{2A}, F_{2B}, F_{3A} and F_{3B} animals were weaned and autopsied comparable to F_{1A} rats, except that randomly selected rats from F_{3A} were used in a chronic feeding study. On gestation day (GD) 20, F_{2C} and F_{3C} litters were exposed by caesarean section. Randomly selected F_0, F_{1B} and F_{2B} parents were kept on the same dietary level for 9 months followed by a histopathological examination. Representative weanlings from F_{2A}, F_{2B}, F_{3A} and F_{3B} also were examined for pathology. All adult rats were weighed weekly and food consumption was measured at the same time. All litters of more than 10 offspring were reduced to 10 by a randomised selection on PND 4. When killed, all adult and weanling rats were autopsied. The treatment had no effect on mortality, but there was a noticeable deterioration in the physical state of rats dosed with 5% via diet, i.e. these rats were smaller and thinner than the controls, and had rough hair coats. Diarrhoea was marked in animals fed the two highest dose levels. The consumption of the diet containing carrageenan was increased at ≥ 2.5% in both sexes. The cumulative weight gain of males at week 12 of the premating period was significantly decreased at the 5% level in F_0, at the three highest dose levels in F_{1B} and at all four dose levels in F_{2B} generation. In females, apart from a significant decrease in the F_{2B} generation at the 5% level, the cumulative weight gain during the premating period was unaffected. For the fertility index, there was no dose-related difference at any dose level. There were no adverse effects concerning average litter size or viability of the pups. The viability index gave no indication for adverse effects of carrageenan and also the average number of liveborn pup surviving to PND 4 indicated no significant postnatal effect at any dose level. The treatment appeared to have no effect on the viability of the pups from birth to weaning at PND 21. Birth weight of the pups were incidentally decreased (2.5 and 5% level F_{1B} female pups and at 5% level F_{3A} male and female pups). Dose-related and statistically significant decreased body weights of weanlings were noted at all dose levels. However, decreased pup weight on PND 21 in the 0.5% dose level group was only significant in the female pups of the F_{1B} litter. Overall, up to the highest dose tested, no effect was observed on reproductive or developmental parameters apart from the effect on pup weight on PND 21. Based on the decreased body weight gain of the male animals and the decreased pup weight at dose levels of ≥ 1%, the authors proposed a NOAEL of 0.5% (equal to about 290 or 350 mg calcium carrageenan/kg bw per day for male or female animals). The Panel agreed with this NOAEL. However, decrease in pup weight was observed in the presence of maternal effects (diarrhoea) which may have affected lactation.

In an one-generation reproductive and behavioural toxicity study with Sprague–Dawley rats, male and female rats were dosed orally with 0–18,000 mg/kg diet of calcium carrageenan (not specified; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30; no indication of molecular weight distribution) (equal to 0, 500, 1,000 or 2,100 mg/kg bw per day for males and for females 0, 400, 700 or 1,500 mg/kg bw per day during the gestation period and 0, 600, 1,300 or 2,600 mg/kg bw per day during the lactation period) (Vorhees et al., 1979). The substance was continuously administered after the acclimation period and included: 14 days prior to mating, 1–4 days during breeding (males were discarded following conception), throughout gestation (22 days), lactation (21 days) and post-weaning testing (69 days), i.e. from weaning at 21 days of age until the termination of the experiment at 90 days of age. Developmental measures in the offspring included physical development (pinna detachment, incisor eruption, eye opening, and growth), behaviour and histopathology of the brain. Calcium carrageenan produced a few isolated and inconsistent effects

(e.g. delay in the appearance of pivoting, advance in the appearance of full locomotion or increase in cell density in the olfactory bulbs); however, there was no clear pattern of behavioural dysfunction and the observed effects were not dose-related. No other fertility or developmental effects were observed although the description of these parameters was limited.

Developmental studies

In all studies described below (Documentation provided to EFSA n. 45), body weights were recorded at regular intervals during gestation and all animals were observed daily for appearance and behaviour. To test the methodology and the sensitivity of the laboratory animals' positive controls were tested. All dams were subjected to caesarean section, and the numbers of implantation sites, resorption sites, live and dead fetuses, and body weights of live fetuses were recorded. All fetuses were examined grossly for sex distribution and for external abnormalities (one-third detailed visceral examination and two-third stained and examined for skeletal defects). In the following developmental toxicity studies, the source and type of carrageenan used were not specified. The Panel interpreted the results as being obtained from carrageenan isolated from *C. crispus* with an approximate ratio ι:κ:λ type 0:70:30. The molecular weight distribution of the tested carrageenan preparations was not indicated.

Mice

Pregnant CD-1 mice (22–27 animals/group) were treated via gavage with 0, 10, 45, 470 or 900 mg/kg bw per day of sodium carrageenan (unspecified) in corn oil (the use of this vehicle was described in Documentation provided to EFSA n. 46) on GD 6–15 (Documentation provided to EFSA n. 45). The dams were sacrificed on GD 17. Mortality was 1, 1, 0, 2 and 9 in the 0, 10, 45, 470 or 900 mg carrageenan/kg bw per day group. The dosing caused an apparent increase in the number of resorptions with or without corresponding decrease in the number of live fetuses in the two highest doses. At the two highest doses, there was a decrease in the fetal weight (approximately 7% and 13%). In these groups also, retardation of skeletal maturation (increased incidence of incomplete or missing sternebrae and incomplete skull closure) was observed. The other soft and skeletal tissue abnormalities observed were comparable to the control group. Due to the high mortality in the high dose group, the Panel considered this study not relevant for risk assessment.

Rats

Pregnant Wistar rats (21–27 animals/group) were treated via gavage with 0, 40, 100, 240 or 600 mg/kg bw per day of sodium carrageenan (unspecified) in corn oil (the vehicle as described in Documentation provided to EFSA n. 46) on GD 6–15 (Documentation provided to EFSA n. 45). The dams were sacrificed on GD 20. The dosing caused an apparent increase in the number of resorption sites with or without corresponding decrease in the number of live fetuses in the two highest dose levels. At the highest dose group, there was a decrease in the fetal weight (15%). In the two highest dose groups also, retardation of skeletal maturation (incomplete or missing sternebrae missing hyoid) was observed. The other soft and skeletal tissue abnormalities were comparable to the control group.

In a follow-up study of the above one (Documentation provided to EFSA n. 46), groups of 21–24 Wistar-derived albino rats were exposed to sodium or calcium carrageenan (unspecified) via diet at concentrations of 0, 1 or 5% (equal to 0, 600 or 3,000 mg/kg bw per day) on GD 6–15. The motivation was that treating by the intubation route entailed a physiological stress and therefore embryo/fetal disorders. The dams were sacrificed on GD 20. No mortalities were observed. For both sodium and calcium carrageenan, the dosing up to 5% caused no adverse effects on maternal or fetal survival, on the number of implantations, or on the degree of maturation of fetuses. The incidence seen of either soft or skeletal tissue defects did not differ between the test groups and the controls. The NOAEL for developmental and maternal toxicity was 5% (3,000 mg/kg bw per day, sodium and calcium carrageenan) the highest dose tested.

The aforementioned three-generation reproductive toxicity study included a developmental part (Collins et al., 1977b). See for details the section reproductive toxicology. The parental animals were F_{1B} and F_{2B} of the three-generation reproductive study, and the offspring were F_{2C} and F_{3C}, respectively. All dams were subjected to caesarean section on GD 20, and the number of corpora lutea, early and late resorptions sites, implantation sites, the live and dead fetuses and the body weight of live fetuses were recorded. All fetuses were examined grossly for external abnormalities, half of the fetuses underwent detailed visceral examinations and the other half were stained and examined for skeletal defects. The average daily high molecular calcium carrageenan (κ-λ) consumption (0%,

0.5%, 1.0%, 2.5% and 5.0%) of the dams of the F_{2C} generation was calculated by the authors to be 0, 309, 609, 1,563 or 3,184 mg/kg bw per day and of the F_{3C} 0, 290, 592, 1,524 or 3,060 mg/kg bw per day, respectively. There were no adverse effects on the average numbers of corpora lutea, implantations, and early or late deaths and the average resorptions (in %) per litter. The Panel noted that there were some incidental inconsistent findings but considered these findings not of biological significance. The Panel considered that the dosing up to 5% (3,060 mg/kg bw per day, the highest dose tested) in the diet gave no indications for maternal abnormalities or fetal external, skeletal and soft-tissue anomalies.

Hamsters

Pregnant Golden hamsters (24–30 animals/group) were treated via gavage with 0, 10, 45, 470 or 900 mg/kg bw per day of sodium carrageenan (unspecified) in corn oil (vehicle as described in Documentation provided to EFSA n. 46) on GD 6–10 (Documentation provided to EFSA n. 45). The dams were sacrificed on GD 14. Mortality was 1, 0, 0, 0, 2 in the 0, 10, 45, 470 or 900 mg sodium carrageenan/kg bw per day group. The number of resorption sites was increased in the 45 mg/kg bw per day group with a corresponding decrease in the number of live fetuses. This effect was not observed at the higher dose levels and therefore not considered to be treatment-related. The treatment had no effect on implantation in the treated groups. At the two highest dose levels, a delayed skeletal maturation (missing sternebrae and incomplete ossification of extremities) and an increased number of malformations (scoliosis, exencephaly and cleft palate) was observed.

In a follow-up study (Documentation provided to EFSA n. 46), groups of 21–26 young adult pregnant Golden hamsters were dosed with sodium or calcium carrageenan (unspecified) via diet at concentrations of 0%, 1% or 5% (calculated by the Panel to be equivalent to 0, 1,000 or 5,000 mg/kg bw per day, Swirsky Gold et al., 1984) on GD 6–10. No adverse effects on maternal or fetal survival, on the number of implantations. The incidence seen of either soft or skeletal tissue defects or skeletal retardations did not differ between the test groups and the controls. In hamsters dosed with 5% calcium carrageenan, there was a reduction in the pregnancy rate, while the dosing of sodium and calcium carrageenan gave no indications for developmental effects. The effect on pregnancy rate may be incidental as it was not observed in the group dosed with sodium carrageenan. Furthermore, dosing was performed after implantation. The Panel considered 5% in the diet (5,000 mg/kg bw per day, sodium or calcium carrageenan, the highest dose tested) as the NOAEL.

Pregnant Golden hamsters (21–26 animals/group) were treated by oral intubation with sodium or calcium carrageenan in distilled water at dose levels of 0, 10, 40, 100 or 200 mg/kg bw per day on GD 6–10 (Collins et al., 1979). On GD 14, one day before expected parturition, the animals were subjected to caesarean section and the numbers of corpora lutea, implantations, resorptions sites, and live and dead fetuses and bodyweights of live pups were recorded. All fetuses were examined grossly for sex distribution and for external abnormalities; approximately half of the fetuses from each litter for detailed visceral examination and half stained and examined for skeletal defects. The dosing with sodium or calcium carrageenan caused no adverse effects in dams and offspring.

Rabbits

Pregnant Dutch-belted rabbits (12–13 animal/group) were treated by intubation one daily from GD 6 to 18 with doses of 0, 40, 100, 240 or 600 mg/kg bw per day sodium carrageenan in corn oil (vehicle as described in Documentation provided to EFSA n. 46) (Documentation provided to EFSA n. 45). Caesarean section was performed on GD 29. Mortality was 4, 3, 0, 2, 3 in the 0, 40, 100, 240 or 600 mg sodium carrageenan/kg bw per day. High mortality was also observed in the control group. The Panel considered this effect was not related to carrageenan but probably to the use of the vehicle (corn oil) or to the general condition of the animals at the start of the study. Live fetuses of each litter were placed in an incubator for 24 h for an evaluation of neonatal survival. Even the highest dose level of 600 mg/kg bw per day caused no effect on implantation on fetal survival. There was also no indication for developmental disorders in either soft or skeletal tissues in treated animals compared with the controls. However, the number pregnant does at caesarean section was low (9, 8, 12, 9 and 7 for the of 0, 40, 100, 240 or 600 mg sodium carrageenan/kg bw per day) and therefore this study cannot be used for risk assessment.

Overall, the Panel noted that the NOAEL for reproductive effects of calcium κ/λ-carrageenan (high molecular) observed in a dietary three-generation reproduction toxicity study in rats was 3,212 mg/kg bw per day, the highest dose tested and 290 mg/kg bw per day for parental and developmental effects based on decreased parental body weight gain and pup weight (Collins et al., 1977a).

Reproductive and neurodevelopmental effects were not observed in a one-generation study in rats up to doses of 2,600 mg sodium κ/λ-carrageenan/kg bw per day (Vorhees et al., 1979). The prenatal developmental studies performed with sodium κ/λ-carrageenan by gavage suspended in corn oil were considered by the Panel not suitable for the safety evaluation (Documentation provided to EFSA n. 45). The Panel considered that the high maternal mortality observed in mice and rabbits was not ascribed to κ/λ-carrageenan but to the vehicle corn oil used and to the method of dosing. The NOAEL for developmental effects found in dietary studies in prenatal developmental toxicity studies was 3,000 and 5,000 mg/kg bw per day sodium or calcium κ/λ-carrageenan for rats and hamsters, respectively (Documentation provided to EFSA n. 46), and 3,060 mg calcium κ/λ-carrageenan/kg bw per day for rats (Collins et al., 1977a) the highest dose levels tested.

The Panel noted that the reproductive and developmental toxicity studies were exclusively conducted with κ/λ- carrageenan and that the molecular weight distribution of the tested carrageenan preparations were not indicated.

No data are available on reproductive and developmental toxicity of ι-carrageenan and processed Eucheuma seaweed.

3.5.7. Hypersensitivity, allergenicity and food intolerance

Animals

Weiner et al. (2015) evaluated the potential effects of carrageenan after 28 days of administration of piglet-adapted formula to Domestic Yorkshire Crossbred Swine (6 males, 6 females per group) from lactation day 3 for 28 consecutive days on several endpoints. The endpoints evaluated included emphasis on the GI tract and the immune system including immunophenotyping, circulating cytokines and immunohistochemistry of the GI tract. Animals were exposed to 0, 300, 1,000 and 2,250 mg/kg diet equal to 0, 52, 193, and 430 mg/kg bw per day and 0, 56, 203, and 448 mg/kg bw per day, for male and female, respectively. The test material sample of κ/λ-carrageenan (FMC Lot. 90303011) (ratio κ/λ unknown) was fully characterised for, molecular weight, percentage of the molecular weight below 50 kDa, known as the low molecular weight tail (LMT) as per European requirements, and met internationally recognised carrageenan food additive specifications. The weight-average molecular weight of the test material sample was 664–732 kDa with a LMT of 0.3–3.9% and a viscosity of 80 cP. Some animals show minor findings, such as faecal changes but clinical pathology parameters, including haematology, clinical chemistry and coagulation were unaffected by the treatment. The results of the immunological evaluations did not reveal any treatment-related changes at the concentrations tested as compared to the vehicle control (swine adapted infant formula).

Humans

The α-1,3-galactosidic linkage, which is present in the three types of carrageenan is specifically recognised by human IgG and IgM anti-galactose natural antibodies. Binding of these antibodies to this saccharide epitope may lead to an immune reaction and, for instance, they were involved in acute rejection by humans of pig xenografts (Galili et al., 1985; Thibaudeau et al., 1996; Klein et al., 1998). These antibodies developed with age in human; they are not present in the neonates but develop within a few months, as soon as the newborn's gastrointestinal tract becomes colonised by microorganisms.

The Panel noted that an ELISA was available for the detection/quantification of carrageenan in various foods (Haines and Patel, 1997, 2005), which indicated that carrageenan could elicit the production of and react with, specific antibodies thus revealing the presence of immunogenic sites in carrageenan. It was not known whether these antibodies also recognised poligeenan and other degraded carrageenans.

Vojdani and Vojdani (2015) reported that 27%, and 17% of the sera from 288 healthy, asymptomatic volunteers contained specific IgG and IgE antibodies to carrageenan, respectively. Fixation of IgE but not of IgG antibodies was largely inhibited by several common food antigens, indicating that these IgE may be formed in response to cross-reacting antigens present in common foods.

The Panel noted that despite the presence in human serum of antibodies capable to recognise saccharidic epitopes on the carrageenan molecules, no case reports of significant allergic and/or anaphylactic reactions after ingestion of foods containing carrageenan were identified in the available literature.

3.5.8. Special studies with carrageenan and degraded carrageenan on the mechanisms of inflammation

A number of studies with samples of carrageenan and 'degraded' carrageenan, which were not always adequately characterised analytically, has been performed in order to evaluate if these compounds were able to induce or increase the production of proinflammatory mediators by various cells. These cell models (mostly intestinal or macrophage-like) were selected by the authors on the ground of their relevance to the oral administration of the additive E 407. Only the most recent ones are reported below as described by their authors.

***In vitro* studies**

Borthakur et al. (2007) reported that when human (intestinal epithelial cells from colonic surgeries, cell line NCM 460) cells, and normal rat ileal epithelial cells were treated for 1–96 h with λ-carrageenan (not analytically characterised) at a concentration of 1 μg/mL, increased BCL10, nuclear and cytoplasmic nuclear factor κB (NF-κB), and IL8 secretion were observed.

In a series of studies with various cell models (RAW 264.7 and 23ScCr mouse macrophage cell lines, NCM 460 human colonic epithelial cell line, primary cultures of human colonic cells), Bhattacharyya et al. (2008b, 2010) studied the mechanisms of carrageenan-induced intestinal inflammation. Toll-like receptor (TLR) and the protein B-cell lymphoma/leukaemia 10 (BCL10) were identified as important components of the cell membrane for binding of carrageenan to the cell membrane. The authors reported that in their studies, binding of carrageenan (purity and composition not reported) to the receptor resulted in stimulation of NF-κB, and activation of interleukin-8 (IL-8) production and of a reactive oxygen species (ROS)-mediated pathway.

Choi et al. (2012) evaluated the effects of carrageenan on proinflammatory transcription factor NF-κB (subfamily RelA/p65) and early growth response gene 1 product (EGR-1) in relation to human intestinal epithelial barrier integrity. Human colonic cancer cell lines HCT-8, HT-29 and Caco-2 were exposed to 0 or 1 μg/mL of carrageenan (type not specified) for up to 24 h. NF-κB activation (but not EGR-1 activation) was involved in the induction of proinflammatory cytokine IL-8. According to the authors, the results showed that both NF-κB and EGR-1 play a role in maintaining the epithelial barrier integrity in response to carrageenan.

Another study assessed 'whether binding of carrageenan to TLR4 was specific or due to the mechanical coating of the membrane as a result of the large molecular weight of carrageenan and of the conditions used *in vitro* (Documentation provided to EFSA n. 47). Human embryonic kidney cells transfected with a TLR4 reporter system were employed to determine the binding, using alkaline phosphatase (SEAP) as the reporter molecule. Cells were exposed for 24 h to various types of carrageenan, all compliant with the JECFA specifications: food-blend carrageenan from a manufacturer, commercial λ, κ and ι-carrageenan, and a commercial mixture of κ- and ι-carrageenan, at concentrations of 0, 0.1, 1, 10, 50, 100, 500, 1,000 or 5,000 ng/mL'. Positive (LPS) and negative (clarified locust bean gum (CLBG) and sodium alginate (SA)) controls were included. Cell viability, as measured by the levels of cellular adenosine triphosphate and released lactate dehydrogenase released, was unaffected by any of the carrageenan or by CLBG, SA or LPS at any exposure concentration tested. Overall, no measurable changes in cell viability were observed under these test conditions, and the three types of carrageenan tested were not TLR4 agonists or antagonists.

McKim et al. (2015) evaluated the ability of the different types of carrageenan (κ-, ι- and ʎ-, food-grade purity 51–85%) to bind and activate TLR4 signalling by using a TLR4/MD-2/CD14/NF-κB/SEAP reporter construct in a HEK293 cell line. The carrageenan used was characterised with respect to identity and purity, and it was observed that commercial carrageenan samples contained sugars (dextrose or sucrose). Cells were exposed for 24 h to concentration of 0.1, 1, 10, 50, 100, 500, 1,000, and 5,000 ng/mL of the various compounds. The results showed that carrageenan did not bind to TLR4 and was not cytotoxic to the HEK293 cells at the concentrations and experimental conditions tested. In addition, it was shown that carrageenan binds tightly to serum proteins, in particular proteins from the fetal serum used in the cell cultures. The Panel noted that the authors claimed that carrageenan was described as having a weight average molecular weight (mw) of 200–800 kDa but that no data presenting the MW profile of the different forms of carrageenan used in the study were reported in this publication.

In another study, McKim et al. (2016) evaluated the intestinal permeability, cytotoxicity and carrageenan-mediated induction of proinflammatory cytokines using a standard Caco-2 absorption model and both ι-, κ- and λ-carrageenan. They found no carrageenan permeability or cytotoxicity at

concentrations of 100, 500 and 1,000 μg/mL. In two human intestinal cell lines (HT-29 and HCT-8) carrageenan (0.1, 1.0 and 10.0 μg/mL) did not induce IL-8, IL-6 or MCP-1 nor produced cellular toxicity at 24 h. The authors concluded that carrageenan did not cross the intestinal epithelial cells and were not cytotoxic to these cells. Carrageenan did not increase cellular oxidative stress nor did they induce expression of proinflammatory genes. Positive control substances produced the expected results indicating that the test systems were responsive. According to the authors, this study was unable to reproduce any of the previously reported *in vitro* findings that carrageenan may cause inflammation or disrupt insulin signalling pathways. They also reported that when a commercial batch of ʎ-carrageenan was carefully characterised for identity and purity, only 64% of the material sold was carrageenan, with 34% being comprised of sugars. The Panel noted that the authors claimed that carrageenan was described as having a weight average molecular weight (mw) of 200–800 kDa but that no data presenting the MW profile of the material used in this study were reported in the publication.

Chan et al. (2017) studied the influence of the three major types of carrageenan polysaccharides on monocyte (human THP1) behaviour *in vitro*, only the λ-type induced monocyte adhesion and only in the presence of serum. Further analyses indicated λ-carrageenan bound IL-8 in the serum and activated the cultured monocytes through an IL-8-dependent pathway.

Fahoum et al. (2017) investigated if 'carrageenan' may modify gastric proteolysis and if 'physiologically (partially) digested carrageenan' (i.e. carrageenan incubated in a simulated gastric fluid (pd carrageenan = pdCGN) may affect gut epithelial structure and function. Food-grade κ-, ι- and λ-types of carrageenan (no indication of molecular weight distribution) were used.

The authors reported that:

- native carrageenan bound milk, soya or egg proteins,
- native carrageenan impaired digestibility by pepsin of these proteins,
- in Caco 2 cells, pdCGNs induced increased expression of inflammatory markers and affected the epithelial structure,
- Some of these effects were associated with the degree of sulfation of the different carrageenan forms, with kappa being less potent.

The Panel noted that:

- the simulated gastric fluid used was close to the 'physiologic' conditions and distinctly different from the treatment used to yield 'polygeenan' or C 16 from carrageenan,
- there was no information on the changes in MW of carrageenan after partial digestion,
- when looking at the biological effects, it was unclear what the partially digested material used in the experiments with 'pdCGNs' was: either 'protein-carrageenan complexes', or 'pure carrageenan' (not in complex with a protein),
- as regards the biological effects (e.g. secretion of mediators of inflammation), there was a difference between the types of pdCGN i.e. iota and lambda having an effect in contrast to kappa. The results with native iota carrageenan were negative.

Therefore, the Panel considered that although the reported effects may have been relevant for this evaluation, it was not possible to use the data from this study.

Applying Caco 2, THP-1 and HT-29 cells as models, the same group (Jiang et al., 2013; Wu et al., 2017), reported the effects of κ-carrageenan on the inflammatory reaction *in vitro*. The results showed that κ-carrageenan could induce significant increase in secretion of various inflammatory cytokines including tumour necrosis factor-α (TNF-α), IL-1β, IL-6, and to participate in the Bcl10-NF-κ B-mediated pathway to enhance LPS stimulated secretion of IL-8 in HT-29 cells. The Panel noted that the MW distribution of the κ-carrageenan used was not given by the authors who only indicated that: 'the average molecular weight of 1×10^6 of kappa carrageenan was used in this experiment' (Wu et al., 2017).

Overall, the interaction of carrageenan with inflammatory pathways at the molecular level was investigated in several *in vitro* cell models. These studies were based on signalling pathways involving the transcription protein nuclear factor κB (NF-kB), which, among others, regulates the expression of genes associated with inflammation. In many studies, the three types of carrageenan (commercial material, data on purity not available) were reported to activate inflammatory cascades that are related to innate immunity and to the generation of ROS, which may lead to inflammation. A recent study conducted with κ-, ι– and λ-carrageenan suggested that the difference in their capacity to react with food and to induce the expression of inflammatory markers may be due to the difference in sulfation of the different forms (Fahoum et al., 2017). The Panel noted that the available *in vitro*

studies with 'carrageenan' were discussed several times (McKim, 2014; Weiner, 2016, 2017). Main pitfalls were that the studies on signalling pathways did not establish neither concentration-related responses, nor direct and specific binding to receptors or entry of carrageenan inside the cells. The author considered that high molecular weight carrageenan might coat the receptors. The Panel acknowledged that the *in vitro* studies are useful to investigate the mechanisms of toxicity. However, the Panel also noted that they were often conducted using established cell lines, which are not fully representative of the normal human intestinal epithelium *in vivo,* and that several confounding factors, including poor characterisation of the material used and limited consideration of the interactions with food were identified in the protocols used.

In vivo studies

Bhattacharyya et al. (2013) investigated the pathways for induction of inflammation in Bcl10 wild-type, heterozygous and null mice. Groups of 3–8 male mice were given drinking-water containing 0 or 10 μg/mL of carrageenan (commercial λ- and κ-types) for 14 weeks, amounting to averaged 50 μg carrageenan/mouse (equivalent to 1.7 mg/kg bw per day). Markers of intestinal inflammation (faecal calprotectin), blood cytokines (including KC, the mouse analogue of human IL-8) and markers of the inflammatory cascade were measured. Body weights were unchanged. Faecal calprotectin and circulating KC were significantly increased in both wild-type and heterozygous mice exposed to carrageenan compared with controls. In all three types of mouse, serum IL-6 and monocyte chemotactic protein-1 (MCP-1) were similarly increased by carrageenan treatment. No treatment-related effects were observed on serum levels of cytokines TNF-α, interferon gamma (IFN-γ), IL-1β, IL-10, IL-12 and IL-23. No gross changes or macroscopic lesions were apparent in the intestine of carrageenan-treated mice, except in one wild-type mouse. Microscopically, in carrageenan-exposed mice, the extent of inflammatory infiltrate throughout the intestine was greater in the wild-type than in the Bcl10 null mice, being significantly greater in the small intestine than in the colon and rectum for each of the groups. IL-10-deficient mice exposed to carrageenan showed an increase in activation of NF-kB (RelA) activation, but no increase in RelB or phospho-Bcl10. According to the authors, these findings demonstrated 'a requirement for Bcl10 to obtain maximum development of the inflammatory pathway by carrageenan and lack of complete suppression by IL-10 of activation of the inflammatory pathway by carrageenan'.

In the study by Shang et al. (2017) C57/BL/6J mice (6/groups) were given normal autoclaved water or λ, κ or ι-carrageenan (purchased from Sigma Shangai; no further precision) in drinking water (20 mg/L) for 6 weeks. Blood (for the estimation of serum TNF-α, IL-1β, IL-6 and IL-10), cecum and colon were collected at the end of the treatment period. A sample of the colon was fixed in 10% formaldehyde then the degree of inflammation was scored after staining with haematoxylin and eosin. The colonic content was collected aseptically for analysis of the microbiota by DNA amplification and high-throughput sequencing. The authors reported that the treatment with carrageenan induced disruption of the epithelium of the colon, colitis, and increased (about three times) the serum TNF-α levels, whatever the type of carrageenan used; serum levels of IL-1β, IL-6 and IL-10 were unchanged. The treatment with λ, κ and ι-carrageenan had different effects on the various components of the gut microbiota but all of them were able to decrease the amount of a bacterium known to have anti-inflammatory properties (*Akkermensia muciniphila*).

Wu et al. (2017) used the *Citrobacter freundii* DBS100-induced intestinal inflammation model with NHS male and female mice to study if κ-carrageenan administered by gavage in water for 1 week at daily doses of 1.7 mg/kg bw, 8.3 mg/kg bw or 41.7 mg/kg bw, could aggravate the inflammatory reaction of the colon to pathogen exposure. They reported that κ-carrageenan modulated cytokine production, down regulated the proportion of T regs and up regulated NF-κB. According to the authors, these results suggested that κ-carrageenan acted as a potential inflammatory agent that could magnify existing intestinal inflammation. The Panel noted that the source of kappa carrageenan used in this study was not indicated. The authors stated that: 'the average molecular weight of 1×10^6 was used in this experiment', but no indication was given about the molecular weight distribution. The Panel also noted that in the absence of LPS stimulation, kappa carrageenan alone did not significantly modify the responses of the cells.

Overall, the Panel noted that the material used for most of the *in vivo* experiments presented was commercial carrageenan, which was however generally not further characterised in particular as regards the amount of low weight-average molecular weight carrageenan, in the region of 50 kDa. In addition, the Panel noted that carrageenan was given in drinking water, which was not representative of the dietary exposure where carrageenan is bound to protein. Therefore, the extent to which these

studies might be representative of the situation when carrageenan is used as a food additive, and consequently the possible use of these studies for the safety assessment of the food additive carrageenan (E 407) where, according to the EU specifications, the amounts of compounds with molecular weight below 50 kDa must be low (< 5%), was deemed limited.

The Panel also noted that intake of carrageenan has been reported to influence the composition of the gastro-intestinal microflora Nilson and Schaller, 1941; Poulsen, 1973; Shang et al., 2017). Interplays between the bacterial gut microflora and sulfated glycans (Mallett et al., 1985; Shang et al., 2016a,b) seem to play a significant role in the development of inflammation in the gut (Pomim, 2017). Because sulfate and undigested sulfur compounds have been implicated in the aetiology of ulcerative colitis (Pitcher and Cummings, 1996; Roediger et al., 1997; Magee et al., 2000; Kamada et al., 2013), the sulfate moiety of carrageenan might be involved in the proinflammatory effects (Pomin, 2015, Fahoum 2017) through the production in the colon of hydrogen sulfide from sulfate, by sulfate-reducing bacteria (Gibson, 1990). Finally, from the reported studies, the purity of the test material used appeared to be of primary importance, and the amount of low molecular weight carrageenan it contained likely influenced the capacity of the test material to induce some effects. However, there is still uncertainty about the 'safe' level of this low molecular weight material. These considerations raised the issue of how the samples used in the studies could be considered as representative of the food additive E 407 as sold in the market. The Panel could not conclude on the potential of carrageenan used as a food additive (E 407) to induce immunotoxic and/or inflammatory effects in the absence of relevant studies using well characterised carrageenan.

3.5.9. Other *in vivo* studies on the effects of carrageenan and degraded carrageenan on the gastro-intestinal tract

Rats

In two consecutive studies (Grasso et al., 1973; Grasso 1975), groups of Wistar rats (males and females) were exposed to carrageenan (5% in diet equivalent to 4,050 mg/kg bw per day) or degraded carrageenan (0.25%, 0.5%, 1% and 5% in drinking water equivalent to 225, 450, 890 and 4,450 mg/kg bw per day) for 21 or 56 days (commercial products for both types of carrageenan were from Glaxo Laboratories, Paris; interpreted by the Panel as isolated from *E. spinosum* with an approximate ratio of ι:κ:λ type 100:0:0). Carrageenan was a fine cream-coloured powder prepared from *E. spinosum* (moisture content, 6%; viscosity of a 0.5% aqueous solution at 25°C, 22 cP; total heavy metals, 20 ppm) and degraded carrageenan was a fine cream-coloured powder prepared by controlled hydrolysis of carrageenan extracted from *E. spinosum* (moisture content, 5%; viscosity of a 0.5% aqueous solution at 25°C 7 cP; sulfate content, 33.2%; total heavy metals, 20 ppm). Rats developed diarrhoea with watery stools when given 5% degraded carrageenan. Only a slight diarrhoea, marked chiefly by faeces which were semi-solid in consistency, was observed in rats given 1% degraded carrageenan or 5% carrageenan. The whole of the GI tract was examined macroscopically for ulceration, but no adverse effects were noted. The Panel considered that the NOAEL for ι-carrageenan was 5% in the diet equivalent to 4,050 mg/kg bw per day, the highest dose tested.

Groups of 10 male and 10 female Sprague–Dawley rats were given either a 5% solution of degraded ι-carrageenan (C16; interpreted by the Panel as low weight-average molecular weight commercial product produced via mild acid hydrolysis of an extract of *E. spinosum* obtained from Laboratories Glaxo, France) as drinking water (daily intake of degraded carrageenan was 6–10 g/kg bw) or a dose of 5 g degraded carrageenan per kg bw in aqueous solution by gavage (once daily; 6 days/wk; 0.5–5 g/kg/bw per day) for 15 months (Fabian et al., 1973). Number-average and weight-average molecular weights for this carrageenan fraction were reported to be in the range of 16–19 kDa and 20–30 kDa, respectively. Groups of 5 rats/sex given distilled water as drinking fluid by gavage served as controls. Rats were necropsied at intervals ranging from 1 to 15 months. At 15 months, treatment of one female and one male which were given degraded carrageenan via drinking water or gavage stopped. These rats were necropsied at 17 and 16.5 months, respectively. After 6 months, squamous metaplasia of the rectal mucosa was reported, together with accumulation of metachromic material (presumed by the authors to be carrageenan) in macrophages. No lesions in the caecum were found, whereas ulcerative lesions in the distal colon could explain the occurrence of occult blood in the stools.

Germfree (n = 9) and conventional (n = 12) female Wistar rats were exposed to degraded carrageenan with a sulfate content of about 30% and an average molecular weight of 20–40 kDa (obtained from Glaxo Labs and interpreted by the Panel produced by mild acid hydrolysis of carrageenan; Hirono et al., 1981). No information on the type of carrageenan is reported. The

degraded carrageenan used in this study was reported as having an average molecular weight of 20–40 kDa. Degraded carrageenan was added at a level of 10% to the basal diet. The germfree rats were necropsied on day 7 (n = 3); day 20, 35 and 63 (n = 2). Three conventional rats (n = 3) were necropsied according the same schedule. Histopathological examination of Swiss-roll preparations of the intestines. Mucosal lesions associated with erosion of the large intestine induced by degraded carrageenan were much more extensive in germfree than in conventional rats.

Six pregnant Wistar rats received drinking water with 5% of degraded carrageenan as (Marcus and Watt, 1971). The carrageenan was derived from the red seaweed *E. spinosum* and was degraded by mild acid hydrolysis to retain a 29% sulfate content, interpreted by the Panel to be mainly ι-carrageenan. No indication about the weight-average molecular weight was given. Six pregnant rats received drinking water without degraded carrageenan. Twelve female offspring from each group were separated and the exposure to 5% degraded carrageenan via drinking water was continued for 6 months. Ulceration of the caecum was observed in 4 of the twelve treated rats. In two of the four rats, the ulceration was accompanied by inflammatory cell infiltration (polymorphonuclear cells and macrophages) and occasionally by glandular hyperplasia at the ulcer margins. In the remaining 2 rats, the ulcers were healed, the mucosa showing atrophy, distortion of glands and fibrosis of the lamina propria.

Degraded carrageenan derived from the red seaweed *E. spinosum* (obtained by mild acid hydrolysis to retain 30% sulfate content and an average molecular weight of 20–40 kDa; interpreted by the Panel to be mainly ι-carrageenan) was given to Sprague–Dawley rats (Wakabayashi et al., 1978). In experiment I, 4 groups of 30 males and 30 females was given a diet with 0%, 1%, 5% or 10% degraded carrageenan *ad libitum* for 24 months (equivalent to 0, 500, 2,500 or 5,000 mg/kg bw per day). In experiment II, 2 groups of 20 males and 20 females was given 0% or 5% aqueous solution of degraded carrageenan *ad libitum* as drinking water for 15 months (equivalent to 0 or 2,500 mg/kg bw per day). In experiment III, 3 groups of 15 males and 15 females were given 0, 1,000 or 5,000 mg degraded carrageenan/kg bw in aqueous solution by gavage for 15 months. Degraded carrageenan induced consecutively: colitis, secondary metaplasia, then tumours (squamous cell carcinomas, adenocarcinomas, adenomas). When degraded carrageenan was given by gavage, the incidence of malignant tumours was lower than when given by diet or drinking water. The Panel noted that this study has been taken into consideration in IARC's evaluation of carcinogenicity of low molecular weight degraded carrageenan (category 2B) (IARC, 1983).

Carrageenan derived from red seaweed *E. spinosum*, and degraded by acid hydrolysis to retain 30% sulfate content, and obtained from Glaxo, France (interpreted by the Panel to be ι-carrageenan; no indication about the weight-average molecular weight was given) was given to male Fischer 344 rats via the diet (10%) for varying periods of time: group I (n = 39) for 2 months (equivalent to 8,100 mg/kg bw per day); group II (n = 42) for 6 months and group III (n = 42) for 9 months (equivalent to 5,000 mg/kg bw per day) (Oohashi et al., 1981). The degraded carrageenan was reported as having an average molecular weight of 20–40 kDa. After termination of the administration on degraded carrageenan, all the rats were given the normal basal diet until 18 months. At that time all rats were autopsied. The controls (n = 46) received normal diet for 18 months. Colorectal squamous metaplasia (100%) and squamous cell carcinomas, anaplastic carcinomas and adenomas (incidence 5/39 animals) were seen after exposure to degraded carrageenan for 2 months. The incidence of colorectal tumours increased with exposure time and was 17/42 (40.5%) after exposure to degraded carrageenan for 9 months. The Panel noted that this study was taken into consideration in IARC's evaluation of carcinogenicity of low molecular weight degraded carrageenan (category 2B) (IARC, 1983).

Guinea pig

In two consecutive studies, Watt and Marcus (1969, 1971) exposed groups of 10 male adult guinea pigs (strain not given) exposed to a 1% drinking solution (not more than 1,500 mg/kg bw per day according to the authors) of ι-carrageenan (commercial product from Cumming & Son Ltd., Salford) via drinking water, or to 5% aqueous drinking solution of degraded carrageenan (commercial product from Laboratoires Glaxo-Evans, Paris; both types interpreted by the Panel as isolated from *E. spinosum* with an approximate ratio of ι:κ:λ type 100:0:0) (not more than 2,000 mg/kg bw per day according to the authors) during 20 or 30 days. No indication about weight-average molecular weight was given. In animals exposed to ι-carrageenan, 2/4 showed ulcerative lesions in the caecum after 20 days and the other 6 animals had lesions after 30 days. No such effects were seen in controls (drinking water). In animals exposed to degraded ι-carrageenan, most of the animals showed looseness of the stools by the end of 10 days; from 20th to the 30th days, all had occult blood in the faeces. Incidence of

ulcerative colitis was 100%. In five animals killed between the 20th and 25th days the lesions were mainly in caecum, while in the remaining animal, killed between the 26th and 30th days, ulceration had extended into the lower colon and rectum. The ulcerations involved mainly the mucosa and showed features of both acute and subacute inflammatory infiltration as well as crypt abscesses.

Multiple caecal ulcerations were noted in guinea pigs (strain not given) dosed with 5% (about 2,000 mg/kg bw per day) carrageenan (mainly ι carrageenan; interpreted by the Panel as isolated from *E. spinosum* with an approximate ratio of ι:κ:λ type 100:0:0) over 2–4 weeks via diet (Sharratt et al., 1970). No indication about the weight-average molecular weight was given. Sequential studies showed that the lesion first develops as an accumulation of macrophages in the lamina propria and subsequently in the submucosa leading to the formation of pale raised areas which can easily be seen macroscopically. Ulceration of the mucosa then occurs, particularly in these areas. The ulcers were small and superficial and affected only the mucosa. A mixed cellular infiltrate, consisting predominantly of macrophages accompanied by polymorphonuclear, lymphocytes, and plasma cells, surrounded the ulcerated area.

In two consecutive studies (Grasso et al., 1973, 1975), white guinea pigs (males and females) were exposed to carrageenan (5% in diet equivalent to 5,900 mg/kg bw per day for rats) or degraded (0.25%, 0.5%, and 2% in drinking water equivalent to approximately 300, 600 and 2,500 mg/kg bw per day) carrageenan for 21–45 days. For both types, commercial products from Laboratoires Glaxo, Paris; interpreted by the Panel as isolated from *E. spinosum* with an approximate ratio of ι:κ:λ type 100:0:0). No indication about the weight-average molecular weight of the tested compound was given. Details of the compounds used are reported in the rat studies described above (Grasso et al., 1973, 1975). Groups of four male animals were exposed to 1% degraded carrageenan in drinking water during 3 weeks and sacrificed week 4, 7, 11, 15. Multiple pin-point caecal and colonic ulcerations were developed after 3–5 week of treatment in guinea pigs given 5% carrageenan in the diet or 2% degraded carrageenan in the drinking water. Ulceration was not observed in the animals receiving degraded carrageenan 0.25% in drinking water. Ulcers appeared as extensive macrophage infiltration at the base, cover a thin layer of fibrin; polymorphonuclear cells and lymphocytes occurred in considerable numbers. Macrophages, polymorphonuclear and lymphocytes deeply infiltrated the epithelium around the ulcer. In some animals, microabscesses were formed close to the ulcerated areas due to substantial polymorphonuclear cell infiltration. No caecal or colonic ulceration was observed in animals sacrificed 1–4 weeks after the end of the treatment, but distinct granulomas with evident carrageenan within the macrophages were observed. In animals scarified at a later stage, the histology of the caecum and colon was unchanged compared to the controls, and no carrageenan could be shown in the macrophages within the lamina propria.

Guinea pigs (8 females of the Hartley strain, average weight 360 g) given 2% partially degraded i-carrageenan (C16) *(*commercial products from Glaxo); interpreted by the Panel as isolated from *E. spinosum* with an approximate ratio of ι:κ:λ type 100:0:0) in sterile distilled drinking water for 2 weeks developed caecal ulceration (Abraham et al., 1974). No indication about the weight-average molecular weight was given. This effect was not observed when the same level of C16 was given in milk, or when lower levels of C16 (0.02% or 0.2%) were given in drinking water for 12 or 10 months, respectively. The authors reported that the ability of macrophage lysosomes in the lamina propria of the guinea pig to endocytose and store C16, which was not seen in the other species, was apparently closely related to the caecal ulceration observed in guinea pigs species.

Engster and Abraham (1976) compared the caecal response of various degraded carrageenan (Marine colloids, 7 ι-fractions of number-average molecular weight between 5 and 145 kDa from *E. spinosum*, 3 κ-fractions of number-average molecular weight between 8.5 and 314 kDa and 3 λ-fractions of number-average molecular weight between 20.8 and 275 kDa from *C. crispus*) administered to female guinea pigs as a 1% solution in the drinking water for 2 weeks. Six ι-fractions were also given to female guinea pigs in the diet at a 2% level for 10 weeks. When administered in the drinking water, all ι-fractions, with the exception of those of lowest (5 kDa) and highest (145 kDa) number-average molecular weights, were absorbed and inflammation, erosion or ulceration were observed in the caecum. By contrast, κ- and λ- carrageenan fractions given in drinking water or ι-fractions given in the diet for 10 weeks produced no caecal damages. According to the authors, these results indicated that caecal ulceration in the guinea pig was caused only by certain molecular weight ι-fractions and when administered in drinking water.

Rabbits

Twenty male Californian rabbits (average body weight 2,950 g) received drinking water with 0, 0.1, 1 or 5% degraded carrageenan derived by mild acid hydrolysis of i-carrageenan from *E. spinosum*

(Glaxo, France, retaining about 29% sulfate) for 6–12 weeks (Watt and Marcus, 1970). No indication about the weight-average molecular weight was given. Animals fed degraded carrageenan at 5% concentration in their drinking water received an average dose of 1,400 mg/kg bw). Diarrhoea associated with occult blood in the faeces developed by the end of 7 days and persisted. All animals of this group showed severe ulceration of the colon. One rabbit fed degraded carrageenan at 1% (average daily dose of 800 mg/kg bw) over a 7-week period developed diarrhoea, occult blood in the faeces was present in all animals after 2 weeks. All animals showed moderate ulceration in the colon. Animals fed 0.1% degraded carrageenan (daily dose of 70 mg/kg bw) for 12 weeks, developed no diarrhoea but occult blood was seen in three rabbits by the end of week 10. Multiple ulcers were found in the colon in three of five rabbits.

Fifteen mature rabbits (not further specified) weighing about 2,200 g and sensitised intramuscularly with 1 mg λ-degraded carrageenan in 1 mL, were administered λ-degraded carrageenan (approximately 30 kDa; not further specified) dissolved in drinking water at a concentration of 1%. No indication about weight-average molecular weight of the tested compound was given. Ten rabbits were given the substance continuously for 12 months and the remaining five were treated for 28 months. They were sacrificed at the end of the treatment period. Histopathological examination showed mild inflammatory changes of the colonic mucosa in all animals and a focal high-grade dysplasia involving the mucosal epithelium in 3/5 animals treated for 28 months. Tumours of the colorectum were not observed (Kitano et al., 1986).

Overall, degraded carrageenan administered in water is known to be a model for the development of colitis in various animal species (Elson et al., 1995). Degraded ι-carrageenan given in drinking water or diet induced ulceration in the caecum and colon of rats, guinea pigs and rabbits. A mixed cellular infiltrate consisting predominantly of macrophages, polymorphonuclear cells, lymphocytes, and plasma cells, surrounded the ulcerated area. Preulcerative changes consisted mainly in accumulation of macrophages in the lamina propria and they were associated with the presence of material that was assumed to be degraded carrageenan in the subepithelial tissue. Rats exposed to degraded ι-carrageenan (weight-average molecular weight reported as 20–30 kDa, or average molecular weight as 20–40 kDa) via drinking water, diet or by gavage developed in first instance colitis, secondary metaplasia and finally tumours (squamous cell carcinomas, adenocarcinomas, adenomas).

3.5.10. Studies reporting other biological effects of carrageenan and degraded carrageenan

In vitro studies

κ- ι-carrageenan (E 407) or a semi-refined κ- (processed Eucheuma seaweed derived from *E. cottonii*) were added to cultured rats hepatocytes and haematopoietic progenitors at concentrations of 0, 1, 10 or 100 μg/L (Parent-Massin et al. 1993). After 20 h of treatment, the morphology and the levels of protein content of hepatocytes, and the lactate dehydrogenase activity revealed no difference compared with the control values for each compound tested. Proliferation of granulo-monocytic progenitors (CFU-GM) was not changed in the presence of κ-processed Eucheuma seaweed and κ- refined carrageenan, while ι- carrageenan induced a light delay in proliferation of haematopoietic progenitors at the highest concentration tested. The authors concluded that these compounds, as well as refined (E 407) and semi-refined (processed Eucheuma seaweed) had no effect in rat hepatocytes *in vitro* and in the rat haematopoietic progenitors clonogenic assay.

Bhattacharyya et al. (2008a) reinvestigated the ability of carrageenan to induce cell death and to decrease cell proliferation, using cells of the NCM460 line derived from normal colonic mucosa, and primary human colonic epithelial cells. λ-carrageenan (from Sigma-Aldrich of unknown source) was used for most of the experiments; cell proliferation was also assessed using commercial κ-carrageenan and ι-carrageenan, as well as degraded carrageenan of molecular weight less than 5 kDa. No indication about the weight-average molecular weight was given. Cells were exposed for 1–8 days to a concentration of 0, 1 or 10 μg/mL of each type of carrageenan. The treatment of cells with λ-carrageenan increased cell death, reduced cell proliferation and caused cell cycle arrest, compared with control cells. There was no indication of apoptosis, and cell death was attributed to necrosis. Different types of carrageenan lead to similar results on cell proliferation. Apart from a dose-related increase in cell death, the results showed comparisons only between controls and 1 μg/mL of λ-carrageenan, therefore it was not possible to determine whether other parameters were also affected in a dose-related manner.

Zainal Ariffin et al. (2014) investigated the cytotoxicity of degraded carrageenan (no further information) and κ- and ι-carrageenan in human intestine and liver cell lines. No indication about weight-average molecular weight of the tested compound was given. Degraded carrageenan was found to inhibit cell proliferation in Caco-2, FHs74 Int, HepG2 and Fa2N-4 cell lines, and the antiproliferative effect was related to apoptosis together with inactivation of cell proliferation genes as determined by morphological observation and molecular analysis. No cytotoxic effect was found with carrageenan towards normal and cancer intestine and liver cell lines.

In the study by Shahrul Hisham et al. (2014), food-grade κ-carrageenan, dried sheet κ-carrageenan, commercial grade κ-carrageenan, food-grade ι-carrageenan and commercial grade i-carrageenan were dissolved in hydrochloric acid and water in order to prepare degraded carrageenan and carrageenan, respectively. The effects of these compounds were assessed using various normal and cancer human intestinal cell lines. Degraded κ-carrageenan inhibited cell proliferation in Caco-2, FHs 74 Int, HepG2 and Fa2N-4 cell lines; on the contrary, carrageenan was not cytotoxic to normal and cancer intestine and liver cell lines.

In vivo studies, including studies in juvenile animals

Mice

Bhattacharyya et al. (2012) investigated a possible relationship with markers related to the development of diabetes following observations on the interaction of carrageenan with TLR4, as systemic inflammation in Type 2 diabetes might be mediated by the TLR4 pathway. Groups of six male mice were administered carrageenan (commercial κ-λ-type, no indication on weight-average molecular weight was given.) in drinking- water at 0 or 10 μg/mL (equal to 1.7 mg/kg bw per day). A glucose tolerance test (GTT) and an insulin resistance test (ITT) were performed in the same mice at 12 and 14 weeks of age, respectively. No treatment-related effects on body weight or water consumption were observed. In the GTT, blood glucose was statistically significantly increased by carrageenan at all time points. In the ITT, blood glucose levels were statistically significant decreased by more than 80% in controls, but by only 43% in the carrageenan-treated mice, at all time points.

The Panel agreed with JECFA who noted a number of limitations in this study: 'the animals underwent an 18-h overnight fast, which is a considerable length of time for mice that are nocturnal feeders and is known to enhance reactions to insulin in mice (Ayala et al., 2010); measurements of blood glucose extended to only 90 min after dextrose injection rather than 120 min, which is recommended as one of the critical time points for comparisons between treated and control groups' (Ayala et al., 2010 as cited in JECFA, 2015).

According to the results of a 1-year study in mice (Bhattacharyya et al., 2015), exposure to carrageenan may lead to fasting hyperglycaemia and in combination with high fat diet exacerbation of glucose intolerance and hyperlipidemia without effect on weight. No indication about the weight-average molecular weight was given.

Rats

Harmuth-Hoene and Schelenz (1980) investigated the effect of polysaccharides, including carrageenan (unspecified type; interpreted by the Panel as isolated from *C. crispus* with an approximate ratio of ι:κ:λ type 0:70:30; Copenhagen Pectin Factory Ltd), given at the 10% level in a semi-synthetic diet (equivalent to 1,200 mg/kg bw per day), on absorption of Ca, Fe, Zn, Cu, Cr and Co, on weight gain and on faecal dry matter excretion, in five groups of 12 weanling male rats each and compared to a control group, over a period of 8 days. No indication on weight-average molecular weight was given. Carrageenan reduced significantly the absorption of all minerals tested and increased both their faecal excretion and that of faecal dry matter.

Minipigs and pigs

A toxicity and toxicokinetic feeding study has been performed on carrageenan in neonatal minipigs and neonatal pigs in accordance with GLP and based on current guidelines and guidance documents for preclinical juvenile studies for drugs (Documentation provided to EFSA n. 32; Documentation provided to EFSA n. 33; also described in Weiner et al., 2015) (for details of the protocol see section 3.5.7. Some animals did show minor findings, such as faecal changes that are not uncommon at this age. The results of the urinalysis did show glucosuria, which was limited to a few animals at 2,250 mg/kg group (1/6 males, 3/6 females). This observation was not considered to have had an adverse impact on the animals affected, as supported by a lack of adverse changes, which included

clinical findings comparable to controls, and good growth and development during the dosing period. In addition, the other functional clinical parameters, including blood glucose, were unaffected by treatment at this dose level and the microscopic evaluation of the kidneys and other tissues did not reveal any changes indicative of the treatment related effect. Therefore, the finding of glucosuria was not considered to be toxicologically meaningful by the authors. The Panel considered the highest dose tested 430 mg/kg bw per day as the NOAEL. The Panel noted that the test compound complied with the EU specifications and noted that glucosuria was also observed in other studies (Bhattacharyya, 2015; USDA, 2016) and needs clarification.

In a piglet model, induction of colitis using 1% carrageenan solution caused intestinal bacterial dysbiosis according to the authors (Munyaka et al., 2016). The solution used in this study was prepared by acid hydrolysis was supposed to contain carrageenan of average molecular weight of Mn 2×10^4 to 3×10^4 as described by the authors. The authors reported that 'certain bacterial shifts observed in the distal GI tract as a result of CG treatment are consistent with previous findings in patients with intestinal bowl disease'. Bacterial diversity was significantly influenced by carrageenan treatment. Especially in the caecum, ascending, and descending colon notable microbial changes in the percentages of the major phyla Firmicutes, Bacteroidetes, and Proteobacteria were induced. Weiner et al. (2017) criticised the description of the carrageenan used in this study and stipulated that the carrageenan used should be mentioned 'degraded carrageenan with a weight average molecular weight (MW) of 20,000–40,000 Da'.

Monkeys

Three groups of three males and five females infant baboons (*Papio cynocephalus*) received infant formula containing nominal concentrations of 0, 300 and 1,500 mg carrageenan/l from birth to 112 days of age (McGill et al., 1977), being the estimated intake about 86 and 432 mg/kg bw per day. The test material was described as native carrageenan (no indication about weight-average molecular weight), it was heated in the formula and bound to protein to stabilise the suspension of fat, protein and other nutrients. Baboons were fed formula five times per day for the first 14 days, four times per day for the next 14 days, three times per day for the next 56 days and twice per day for 28 days until 112 days of age. No other food or fluid were given to the animals. The baboons were observed daily and the body weight was measured daily. Stools were checked weekly for occult blood. Blood samples were taken monthly, and organ weights were measured at necropsy. On the night before necropsy, each baboon was given water only *ad libitum*. At necropsy under anaesthesia, the GI tract was removed and fixed in 10% buffered formalin. The gut was observed minute-by-minute as fresh and after fixation. Samples were taken for both histological section and for microscopic analysis (from all major viscera, muscle, lymph nodes and the central nervous system). Microscopic examination of the colon showed reddening areas beneath the epithelium and to a lesser degree deeper in the *lamina propia* on which in few instances, deposits of granular brown pigment representing hemosiderin were reported. Occult blood in the faeces was also reported. Although the authors could not explain the cause or origin of the occult blood in faeces they concluded that it was not associated with carrageenan content of the formula. There were no effects on: organ or body weights, urine and faeces, haematological or clinical chemical variables. Hyperplasia of lymphatic tissue and the inflammatory lesions of the colon were present in all animals, regardless of the presence or concentration of carrageenan. The authors attributed these findings to possible antigenic stimulation by exogenous milk proteins.

3.5.11. Human data

Infants and children

Sherry et al. (1993; letter to the journal editor) assessed whether there was a correlation between the frequency of symptomatic upper respiratory tract infections and the type of formula during the first 6 months of life in full term-infants. No indication about weight-average molecular weight was given. In this study, 1,269 infants given liquid formula containing carrageenan at 300 mg/L (0.03%) were compared with 149 infants given powder-based formula without carrageenan. A slightly higher proportion of infants (53.4%) given liquid infant formula containing carrageenan were illness-free during the first 6 months of life compared to those receiving powder-based formula without carrageenan (47.6%). A slight difference in the number of months in which at least one upper respiratory tract infection occurred was also found in favour of the infants receiving liquid formula (0.9 month) compared to powdered formula (1 month). However, the differences between the two groups

were no statistically significant. Based on the results, the authors suggested that the carrageenan-containing liquid infant formula was not immunosuppressive. The Panel considered that the details of the study reported in the letter to the journal were too limited to draw a reliable conclusion.

Another masked, randomised, parallel growth and tolerance study was conducted on healthy infants (aged 0–9 days at the time of enrolment) during the first four months of life (Borschel et al., 2002). A total of 195 infants were given, from enrolment until 112 days of age, extensively hydrolysed casein–based formulas in two different forms: powdered (PWD) that did not contain carrageenan (95 infants), and liquid ready-to-feed (RTF) containing carrageenan (100 infants). No indication about the weight-average molecular weight was given. One hundred and thirty-seven infants completed the study, whereas 58 exited the study in early stages. Among these, 21 subjects from the RTF group and 16 from the PWD group left the study due to intolerance symptoms. Intake, stool patterns and anthropometric measurements (e.g. weight, length and head circumference) were monitored at entry and on days 14 (only anthropometric parameters), 28, 56, 84 and 112 of the study. Overall, no statistically significant differences between the two groups were reported in weight gain, length, head circumference or tolerance to the formulas.

Adults

Bonfils (1970) did not report any evidence of additional gastrointestinal disease in any of the 200 patients with peptic ulcers treated with Ebimar (degraded carrageenan). In a further experiment designed to determine whether degraded carrageenan is absorbed from the colon in patients with ulcerative colitis, 10 such patients were given 10 g Ebimar for 10 days, equivalent to twice the therapeutic dose. No absorption could be demonstrated at this dosage and regular sigmoidoscopic examinations revealed no adverse changes with respect to colonic ulceration or mucosal bleeding. The author concluded that, based on his 12 years experience of the clinical use of degraded carrageenan, as well as the toxicological and pharmacological investigations he carried out, in human beings the therapeutic use of degraded carrageenan is free from side-effects and from risk of toxicity to the colon.

No adverse effects have been described after pharmaceutical uses of degraded carrageenan.

A randomised, double-blind, placebo-controlled, multicenter, clinical trial was conducted to assess if patients with ulcerative colitis in remission would have a longer interval to relapse if they followed a diet without carrageenan (Bhattacharyya et al., 2017). No indication about the weight-average molecular weight of the tested material was given. It is reported that most of the food-grade carrageenan is comprised of κ- and λ-carrageenans of molecular weight 200–800 kDa. Twelve patients completed the study questionnaires. There was relapse in three patients who received carrageenan-containing capsules. None of the patients who received placebo-containing capsules relapsed ($p = 0.046$, log-rank test). Laboratory tests showed increases in faecal calprotectin ($p = 0.06$; paired t-test, two-tailed) and interleukin-6 ($p = 0.02$, paired t-test, two-tailed) between the beginning and the end of study participation in the carrageenan-exposed group, but not in the placebo group. The authors concluded that carrageenan intake contributed to an earlier relapse in patients with ulcerative colitis in remission. The Panel noted that the study is of limited relevance due to the low number of patients involved.

3.6. Discussion

The present opinion deals with the re-evaluation of the safety of food-grade carrageenan (E 407) and processes Eucheuma seaweed (E 407a) used as food additives.

According to the Commission Regulation (EU) No 231/2012[1], 'carrageenan (E 407) is obtained by extraction with water or dilute aqueous alkali of strains of seaweeds of *Gigartinaceae*, *Solieriaceae*, *Hypneaceae* and *Furcellariaceae*, families of the class Rhodophyceae (red seaweeds). It consists chiefly of the potassium, sodium, magnesium and calcium sulphate esters of galactose and 3,6-anhydrogalactose polysaccharides. These hexoses are alternately linked α-1,3 and β-1,4 in the copolymer. The prevalent polysaccharides in carrageenan are designated as κ-, ι-, λ- depending on the number of sulphate by repeating unit (i.e. 1,2,3 sulphate)'.

Processed Eucheuma seaweed (E 407a) is, according to the Commission Regulation (EU) 231/2012, 'obtained by aqueous alkaline (KOH) treatment at high temperature of the strains of seaweeds *E. cottonii* and *E. spinosum*, of the class *Rhodophyceae* (red seaweeds) followed by fresh water washing to remove impurities and drying to obtain the product' complying with the EC specifications. Up to 15% algal cellulose is also present in the product. The main polysaccharide is κ-carrageenan.

Because of the structural similarities of processed Eucheuma seaweed and the conventionally processed food-grade carrageenan and the similarities of effects they caused in the comparative study (Documentation provided to EFSA n. 38), the Panel concluded that the re-evaluation of processed Eucheuma seaweed (E 407a) can be included in that of food-grade carrageenan (E 407).

According to one of the interested parties (Documentation provided to EFSA n. 19), carrageenan has a molecular weight distribution from 30 kDa to as high as 5,000 kDa and is defined as having a weight-average molecular weight between 200 and 800 kDa. Furthermore, it has been confirmed by industry (Documentation provided to EFSA n. 19), that commercial carrageenan (E 407) may have a weight-average molecular weight as low as 200 kDa. In view of the Panel, the molecular weight distribution of such a carrageenan product may have a considerable fraction of molecules encompassing weight-average molecular weight of degraded carrageenan.

The Panel noted that in the literature the information on the molecular weight in the individual carrageenan preparation is often unprecise mixing the terms 'molecular weight', 'weight-average molecular weight' and 'number-average molecular weight'.

Uno et al. (2001a), in a survey of 29 samples of food-grade carrageenan representing κ-, ι- and λ-carrageenan determined a number-average molecular weight of 193–324 kDa and a weight-average molecular weight of 453–652 kDa. The molecular weight distributions of 29 samples of food-grade purified carrageenan were studied by combined gel permeation/inductively coupled plasma (GPC/ICP) method and 'no obvious peak of poligeenan was detected'. According to Uno et al. (2001a), the 'detection limit' for poligeenan was about 5% in the sample of carrageenan.

Validated analytical methods to accurately measure the low molecular weight distribution of carrageenan appear to be not fully developed or available to all industries (Cohen and Ito, 2006; Documentation provided to EFSA n. 15). According to information from one of the interested parties (Documentation provided to EFSA n. 19), 'in native carrageenan, this LMT region represents less than 10% of the total carrageenan molecule…Although there is a Commission specification of not more than 5% at less than 50 kDa, in practice there is no validated analytical method that can accurately quantify the percent of LMT present'.

The Panel noted that, according to the interested parties, an inter-laboratory validated analytical method to accurately measure the low molecular weight distributions of carrageenan is not fully developed or available at present.

The debate in literature with respect to the safety of native (undegraded) carrageenan has been related to the presence of degraded carrageenan. Degraded carrageenan is also known in forms of artificially produced product from carrageenan (e.g. poligeenan, C16) associated with adverse effects. Poligeenan is a much lower weight-average molecular weight polymer (10–20 kDa) generated by subjecting ι-carrageenan to the extreme conditions of acid hydrolysis at low pH (0.9–1.3) and high temperatures (> 80°C) for several hours (Cohen and Ito, 2006; McKim, 2014; JECFA, 2015; USDA, 2016). C16 is artificially formed from ι-carrageenan under conditions of acidic hydrolysis with 0.1 M sulfuric acid, at 60°C for 1.5 h (Glaxo Lab). Contradictory statements in the literature on carrageenan/degraded carrageenan stem largely from inadequate descriptions of the experiments performed and the results obtained in these experiments, and lead to confusion (Benitz et al., 1973).

The Panel emphasised that degraded carrageenan (e.g. poligeenan or C16) has not been authorised as a food additive in the EU. Information available from the US indicated that poligeenan is not used in any food applications (USDA, 2016). The Panel further noted that the term 'poligeenan' has been used in literature including test protocols in a broader sense. However, more specifically poligeenan is a breakdown product of ι-carrageenan consisting of the same sulfated galactose and sulfated anydrogalactose units, linked by α-1,3- and β-1,4-glycosidic bonds as in all types of carrageenan (see Figure 1). According to Figure 2 (Documentation provided to EFSA n. 18), poligeenan comprises polysaccharide molecules of molecular weight ranging from approximately 2 to 200 kDa, and the weight-average molecular weight of poligeenan ranges from 10 to 20 kDa. The Panel noted that based on the data provided (Documentation provided to EFSA n. 18) the overlapping between the low molecular weight tail of carrageenan and high molecular weight tail of poligeenan is increasing with the decrease of the weight-average molecular weight of the carrageenan preparation.

The Panel noted that in most toxicity studies no adequate information on the molecular weight distribution of carrageenan was available. Therefore, it was not possible to assess if the carrageenan tested complied with the EU specifications (specially the limit of low molecular weight carrageenan < 5% below 50 kDa). Furthermore, the Panel noted the methodological limitations of *in vivo* studies, in which carrageenan was given in drinking water, which is not representative of the dietary exposure where food-grade carrageenan will be bound to protein. Therefore, the extent to which drinking water

studies might be representative for food-grade carrageenan used as a food additive and consequently of the relevance of these studies for its safety assessment was deemed limited.

In several *in vivo* studies in rats, guinea pigs, minipigs, pigs and monkeys faecal excretion of high molecular weight carrageenans were shown to be 98–100% of the ingested amount. These studies included rats with normal gut microflora and rats with their gut microflora replaced by human faecal microflora. Several short-term and long-term studies performed in rats dosed via diet containing different high molecular weight types of carrageenan confirmed the absence of tissue storage of these types of carrageenan. However, degraded carrageenan like C16 or low molecular weight carrageenan (number-weight molecular weight of 88 kDa or less) have been described to be absorbed and to be present in various tissues, namely the liver, and the urine of animals given degraded carrageenan, mainly when given in drinking water but also when administered via the diet. Due to the high percentage of administered high weight-average molecular weight carrageenan recovered in faeces, its degradation to lower weight-average molecular weight carrageenan should be very limited. The Panel noted that the conditions (duration, pH, temperature) for potential hydrolysis of carrageenan in the GI tract are less extreme than the 'mild' acidic conditions which resulted in the formation of chemically degraded carrageenan (C16). The theoretical possibility that more limited degradation could occur under conditions representative of the *in vivo* situation remains an uncertainty although this has not been observed to any significant extent in the *in vivo* ADME studies reported.

The Panel further noted that the tested carrageenan differed in their molecular weight distribution, and the Panel considered that existing results did not allow to evaluate kinetic differences between the different types of native carrageenan (κ-, λ- and ι-) and their corresponding low weight-average molecular weight fractions.

The acute toxicity of carrageenan was low.

Short-term and subchronic administration of carrageenan was well tolerated and induced no adverse effects in the gastrointestinal tract of mice, rats, guinea pigs, pigs and monkeys in the majority of studies. However, one study in rats (Morgan and Joiner, 1975) receiving commercial carrageenan reported diarrhoea in all dosed animals accompanied by the excretion of bloody stools in male and female rats at the highest dose tested. The Panel considered 4,500 mg/kg bw per day as the NOAEL for short-term and subchronic administration of carrageenan and processed Eucheuma seaweed. This was the highest dose tested in a subchronic oral toxicity study in rats. Also in a 90-day study in rats, performed according to the OECD guidelines with κ-carrageenan with an average molecular weight range of 196–257 kDa (not specified if number- or weight-average molecular weight) almost complying with the EU specifications (LMT of 1.9–12% (mean 7%) < 50 kDa) the NOAEL was the highest dose tested (3,394 mg/kg bw per day in males, and 3,867 mg/kg bw per day in females) (Weiner et al., 2007).

Monkeys given degraded ι-carrageenan (C16) for 7–14 weeks in drinking water showed histopathological lesions in the colon, which varied from slight mucosal erosions at the low dose (750 mg/kg bw per day) to ulceration associated with inflammatory infiltration of the lamina propria at the high dose (2,900 mg/kg bw per day). In this study, all monkeys on C16 lost blood frequently from the intestinal tract in a dose-related degree and developed some degree of anaemia (Benitz et al., 1973). The Panel considered 750 mg degraded ι-carrageenan (C16)/kg bw per day the LOAEL. Guinea pigs given degraded ι-carrageenan via drinking water developed caecal ulceration. This was not seen when degraded ι-carrageenan was given via the diet. Fractions of degraded κ- and λ-carrageenan given to guinea pigs either via drinking water or diet did not cause gastrointestinal tract damages. The Panel noted, that based on the 'nearly identical molecular structure as carrageenan' and 'similar weight-average molecular weight' poligeenan has been used in experimental studies, as a surrogate for the LMT of carrageenan (Weiner et al., 2015).

Carrageenan (different types) has been tested for genotoxicity in several *in vitro* and *in vivo* test systems. For calcium carrageenan severe adverse effects (aberrations in the anaphase chromosomes of human embryonic lung WI38 cells) were reported in an *in vitro* study. However, when tested *in vivo* (host-mediated assay with male mice and dominant lethal test in rats), calcium carrageenan was considered to be non-mutagenic. The Panel considered that there was no evidence for a genotoxic potential of carrageenan.

Processed Eucheuma seaweed derived from either *E. cottonii* or *E. spinosum* was not mutagenic in a bacterial reverse mutation assay. The negative studies (bacterial reverse mutation test, bone marrow micronucleus test, host mediated assay) performed by Sylianco et al. (1993) were considered by JECFA to be inadequate. However, JECFA concluded that no further studies of genotoxicity were required. The Panel agreed with this conclusion.

Degraded carrageenan showed no evidence of unscheduled DNA synthesis in studies for genotoxicity using DNA repair tests with cultured rat hepatocytes and intestinal mucosal cells. Degraded carrageenan was not mutagenic in the Salmonella mutagenicity test. These observations suggested that the carcinogenicity of degraded carrageenan observed in two rat studies (Wakabayashi et al., 1978; Oohashi et al., 1981) and leading to an IARC classification 2B (IARC, 1983), was most probably due to a non-genotoxic (thresholded) mechanism (Mori et al., 1985).

Overall, the Panel concluded that carrageenan and processed Eucheuma seaweed did not raise a concern with respect to genotoxicity. The Panel further noted that degraded carrageenan was not of genotoxic concern; this further supported that the food additives E 407 and E 407a, even if they contain some low molecular weight carrageenan, were not of genotoxic concern.

Several chronic toxicity studies in rats have been performed with carrageenan (mostly κ/λ-type, molecular weight distribution not adequately described). From the available rat studies, NOAELs up to 7,500 mg/kg bw per day, the highest dose tested, were identified (Nilson and Wagner, 1959). However, in another study in rats given carrageenan preparations with a molecular weight of 244 kDa and 252 kDa, a LOAEL of 1% in the diet (equivalent to 500 mg/kg bw per day) was identified by the Panel (Documentation provided to EFSA n. 43). The Panel noted that the characterisation of the test material in all the chronic studies was limited.

In Rhesus monkeys dosed with a commercial carrageenan composed of κ/λ-carrageenan types at a ratio of 70:30 (from *C. crispus*) amounting up to 500 mg/kg bw per day for 7.5 years, occult blood was observed in treated animals (Abraham et al., 1983). Furthermore, faeces of monkeys given carrageenan were found to contain degraded carrageenan. Since the faeces of monkeys given carrageenan were found to contain degraded carrageenan, the Panel noted that it cannot be excluded that the presence of occult blood in animals is treatment-related.

Overall, the Panel considered that the information available suggested that the colon haemorrhagic properties reported in some studies having tested carrageenan, could be associated with the presence of low molecular weight carrageenan in the test preparation. Therefore, the particular low molecular weight range of degraded carrageenan should be avoided in the specifications for the food additives carrageenan (E 407) and processed Euchema seaweed (E 407a).

From the results of the chronic toxicity studies, which were mostly conducted with κ/λ-carrageenan, the Panel considered that food-grade carrageenan (E 407) was not carcinogenic.

In dietary prenatal developmental studies, no developmental effects were observed at 3,000 and 5,000 mg/kg bw per day for sodium or calcium carrageenan (κ/λ-type) for rats and hamsters (Documentation provided to EFSA n. 46), and at 3,060 mg calcium carrageenan/kg bw per day for rats (Collins et al., 1977a), the highest doses tested. The Panel noted that the NOAEL for reproductive effects of calcium κ/λ-carrageenan, (no indication of molecular weight distribution) observed in a dietary three-generation reproductive toxicity study in rats was 3,212 mg/kg bw per day, the highest dose tested, and 290 mg/kg bw per day for parental and developmental effects, based on decreased parental body weight gain and pup weight (Collins et al., 1977a). Reproductive and neurodevelopmental effects were not observed in one-generation study in rats up to doses of 2,600 mg sodium carrageenan/kg bw per day, the highest dose tested (Vorhees et al., 1979).

The Panel noted that testing for chronic toxicity and reproductive and developmental toxicity was performed almost exclusively with κ/λ-carrageenan of which the molecular weight distribution was not or not adequately described. That ι-carrageenan is rarely characterised for these endpoints is noted against the well known inflammatory effects of degraded ι-carrageenan, which was shown to induce colitis and finally tumours in the rat.

In a well-conducted feeding study with κ/λ- carrageenan (test substance (ratio κ/λ unknown), fully characterised for molecular weight, percentage of molecular weight below 50 kDa, known as LMT as per European requirements) in neonatal pigs (Domestic Yorkshire Crossbred Swine, 6 males, 6 females per group), administration in piglet-adapted formula from lactation day 3 for 28 consecutive days), the Panel identified a NOAEL of 430 mg/kg bw per day, the highest dose tested. The weight-average molecular weight of the test material sample was 664–732 kDa with a LMT of 0.3–3.9%. No effects were reported on various parameters exploring the immune system (Documentation provided to EFSA n. 33; Weiner, 2015).

In two brief reports on studies in human infants, given either a formula containing carrageenan at 300 mg/L or a formula for special medical purposes containing carrageenan at 1,000 mg/L, no differences in comparison to the control groups were described.

The available database on processed Eucheuma seaweed was limited to the endpoints of subchronic toxicity and genotoxicity but was sufficient for the evaluation of the similarities between processed Euchema seaweed and food-grade carrageenan.

In 1974, JECFA used the results of a chronic (life-time) dietary study in rats (Nilson and Wagner, 1959) to allocate an ADI of 75 mg/kg bw per day based on a NOAEL of 15% carrageenan in the diet (equal to 7,500 mg/kg bw per day). In 1978, the SCF endorsed this value and confirmed it in the latest evaluation in 2003. The Panel, however, noted several flaws in that study (use of different lots of carrageenan, presence of high levels of arsenic in the test material), and lack of agreement with the current guidelines for long term studies used for the hazard characterisation.

The Panel noted that the α-1,3-galactosidic linkage, which is present in all the carrageenan molecules is specifically recognised by human anti-Gal IgG and IgM antibodies. Binding of these natural antibodies to this saccharide epitope may lead to an immune reaction and, for instance, are involved in acute rejection by humans of pig xenografts (Galili et al., 1985; Thibaudeau et al., 1996; Klein et al., 1998). The Panel noted that these antibodies developed with age in humans; they are absent in the neonates but develop within a few months, as soon as the newborn's gastrointestinal tract becomes colonised by microorganisms. The Panel noted that despite the presence in human serum of antibodies capable to recognise saccharidic epitopes on the carrageenan molecules, no case reports of significant allergic and/or anaphylactic reactions after ingestion of foods containing carrageenan were identified in the available literature.

The Panel noted that acid degraded ι-carrageenan (C16 and poligeenan), which are clearly different from the food-grade additive E 407, have been often used to induce inflammations and ulcerations in the intestinal tract of rats, guinea pigs and rabbits, in order to develop an experimental model of human inflammatory bowel disease (Watt and Marcus, 1971; Elson et al., 1995; Martino et al., 2017).

Tobacman (2001) published a review on the association between exposure to carrageenan and the development of colonic ulcerations and gastrointestinal neoplasms in animal experiments. This author suggested that (native) undegraded carrageenan can be degraded to 'poligeenan' via acid hydrolysis in the stomach or by enzyme digestion in the intestines or by the microbiota of the gut. Critiques of the paper noted that Tobacman ascribed results of animal feeding studies with poligeenan to carrageenan and disregarded how the method of administration could affect the results (Watson, 2008). Weiner (2014) published a critical review of the available *in vivo* safety studies in which the author considered that existing studies on immune system parameters were conducted mainly with κ-carrageenan in drinking water, which provided data that were not directly applicable to dietary exposure, where carrageenan is tightly bound to protein. According to this author, based on the available studies conducted with carrageenan in feed, carrageenan has not been shown to produce immunotoxicity. The Panel agreed with this conclusion.

In many of the studies reporting the development of an inflammatory reaction after ingestion of 'carrageenan', the material used was not clearly characterised analytically and in particular, the distinction was not undoubtedly made between 'native carrageenan', degraded carrageenan C16 (material prepared to contain a significant amount of low molecular weight material), and poligeenan (ι-carrageenan treated under specific conditions of temperature and pH to fully hydrolyse high molecular weight carrageenan). These weaknesses in the identification of the test sample resulted in confusion in the interpretation of the data.

The Panel noted that intake of carrageenan has been reported to influence the composition of the gastrointestinal microflora (Nilson and Schuller, 1941; Poulsen, 1973; Shang et al., 2017). Interplays between the bacterial gut microflora and sulfated glycans (Mallett et al., 1985; Shang et al., 2016a,b) seem to play a significant role in the development of inflammation in the gut (Pomim, 2017). Because sulfate and undigested sulfur compounds have been implicated in the aetiology of ulcerative colitis (Pitcher and Cummings, 1996, 1996; Roediger et al., 1997; Magee et al., 2000, 2000; Kamada et al., 2013), the Panel considered that the sulfate moiety of carrageenan might be involved in the proinflammatory effects (Pomin, 2015; Fahoum, 2017) through the production in the colon of hydrogen sulfide from sulfate, by sulfate-reducing bacteria (Gibson, 1990).

Before reaching a definitive conclusion on the possible inflammatory and immunotoxic effects of carrageenan used as a food additive, the Panel considered that these effects and the presence in human plasma of natural antibodies, which recognised galactose with a α-1,3-galactosidic linkage such as present in carrageenan, deserve further investigation.

In the view of the Panel, results from a limited study, suggesting that carrageenan intake may contribute to an earlier relapse in patients with ulcerative colitis in remission compared to untreated controls, need further investigation.

The Panel also noted, that experimental results of *in vitro* and animal studies showing a possible influence of carrageenan exposure on glucose tolerance need clarification.

Taking into consideration the lack of adequate data and the existing uncertainties, the Panel was unable to establish a new ADI and decided to change the existing ADI to a temporary ADI, which should only be maintained until the data gaps are addressed.

For the food additives E 407 and E 407a, the fraction of low molecular weight carrageenan (< 50 kDa) is limited in the EC specifications by the purity criteria. This fraction has been associated with potential health hazards similar to those reported for preparations of degraded carrageenan such as poligeenan, to which this fraction shows similarity in molecular structure and in weight-average molecular weight. No data are available to demonstrate that poligeenan shows also similarities in conformations. Against this background, the Panel emphasised the need to establish a fully interlaboratory validated analytical method to detect low molecular weight carrageenan in the food additives E 407 and E 407a at the given limit. To overcome the limitation of the available analytical methods, the possibility of extending the molecular weight of the existing 5% limit for low molecular weight carrageenan from 50 kDa to up to 200 kDa may be considered. Furthermore, in the definitions for carrageenan (E 407) and processed Eucheuma seaweed (E 407) in the Commission Regulation (EU) No 231/2012 the weight-average molecular weight range should be specified in a narrow way avoiding a significant overlap with the molecular weight range of poligeenan.

To assess the dietary exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives, the exposure was calculated based on (1) maximum use levels provided to EFSA (defined as the maximum level exposure assessment scenario) and (2) reported use levels (defined as the refined exposure assessment scenario, brand-loyal and non-brand-loyal).

Carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised in a wide range of foods. The Panel did identify brand loyalty for specific food categories, e.g. flavoured fermented milk products for infants and toddlers, thus the Panel considered this scenario as the most appropriate and realistic scenario for risk characterisation for all population groups.

A maximum estimated exposure assessment scenario taking into account the food for special medical purposes for infants and young children (FC 13.1.5.1. Dietary foods for infants for special medical purposes and special formulae for infants, and FC 13.1.5.2. Dietary foods for babies and young children for special medical purposes as defined by Commission Directive 1999/22/EC) was also performed using food industry data to estimate exposure for infants and toddlers who may be on a specific diet. This exposure scenario considered products belonging to food categories 13.1.2 and 13.1.4 excluding food category 13.1.1 (infant formulae) and 13.1.3 (processed cereal-based foods) where carrageenan (E 407) and processed Eucheuma seaweed (E 407a), according to EU regulation, are not authorised. Considering that this diet is required due to specific needs, it is assumed that consumers are loyal to the food brand, therefore, the maximum reported use level for the FSMP was used and the mean of the typical reported for the remaining food categories.

A refined estimated exposure assessment scenario taking into account the consumption of food supplements was also performed to estimate exposure for children, adolescents, adults and the elderly, for consumers only, as exposure via food supplements may deviate largely from that via food, and the number of food supplement consumers may be low depending on the population and survey.

The refined estimates were based on 41 out of 79 food categories in which carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised. Overall, the Panel considered that the uncertainties identified would, in general, result in an overestimation of the real exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives in European countries for both the maximum level exposure scenario and for the refined exposure assessment scenarios when considering only food additives uses for which data have been provided.

No information on the usage levels of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) has been reported to EFSA for 19 food categories. However, the Panel noted that, given the information from the Mintel GNPD, it may be assumed that carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are used in several food categories (n = 7) for which no data have been provided by food industry. The Panel noted that out of these 19 food categories, pasta and breakfast cereals are products highly consumed. If this would be confirmed, it would therefore result in an underestimation of the exposure.

A possible additional exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use directly by the consumer in adding special powder to thicken foods (soup, sauce, cream, dessert, pap for children) and from their use as food additives authorised in accordance with

Annex III to Regulation (EC) No 1333/2008 were not considered in any of the above exposure assessment scenarios.

The Panel also noted that the refined exposure estimates were based on information reported on the use levels of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) by food industry. If actual practices change, these refined estimates may no longer be representative and should be updated.

For FSMP, the Panel noted that one industry reported a use of carrageenan as food additive at the level of the MPL (300 mg/kg), according to the Annex II to Regulation (EC) No 1333/2008, in one niche product which is not align with information reported from the Mintel GNPD (Appendix D), where no FSMP products seem to be labelled on the market. If this information is confirmed, the uncertainties arising from the FSMP exposure scenario would lead to an overestimation of the current exposure to carrageenan products as food additives calculated by the Panel.

The present re-evaluation included the use of carrageenan (E 407) in foods for infants from 12 weeks of age and for young children.

In 2007, JECFA reviewed all the available toxicological data and concluded that the group ADI 'not specified' for the sum of carrageenan and processed Eucheuma seaweed was maintained for food additive uses in foods other than infant formula (JECFA, 2007b). In its latest evaluation in 2015, JECFA reviewed data published since 2007 and concluded that 'the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1,000 mg/L is not of concern' (JECFA, 2015).

The SCF maintained the original group ADI for food-grade carrageenan and processed Eucheuma seaweed of 0–75 mg/kg bw because of some uncertainty 'about the general immuno-reactive potential of the various carrageenan now in use as food additives' (SCF, 1996).

Concerning uses of carrageenan in food for infants and young children the Panel concurred with the SCF (SCF (1998a, 2003a,b) 'In the absence of any further information on possible absorption of carrageenan by the immature gut in the very young infant, the Committee reaffirms its earlier view (SCF, 1998a) that it remains inadvisable to use carrageenan in infant formulae that are fed from birth, including those in the category of foods for special medical purposes. The Committee has no objection to the use of carrageenan in foods for older infants, such as follow-on milks (SCF, 1983) and weaning foods (SCF, 2003a)'. 'The Committee further recommends maintaining the concept that if more than one of the three substances locust bean gum, guar gum or carrageenan are added to a follow-on formula, the maximum level established for each of those substances is lowered with that relative part as is present of the other substances together'.

Concerns have been raised regarding possible a greater sensitivity of infants to gastrointestinal effects of carrageenan when compared to adolescents or adults (Koletzko et al., 2005). The Panel noted that colitis was reported at high doses or after prolonged administration that were not comparable to the exposure expected from the use of carrageenan as a food additive. Furthermore, it was not clear from the reports whether the carrageenan used in these studies complied with the EU specifications. However, the Panel considered that the available data were not sufficient to rule out a possible increased sensitivity of infants to colitis induction by carrageenan.

4. Overall considerations and conclusions

In its evaluation of carrageenan (E 407) and processed Eucheuma seaweed (E 407a), the Panel noted that:

- according to industry carrageenan (E 407) is defined as having a weight-average molecular weight of 200–800 kDa;
- Uno et al. (2001a) determined in a survey on samples of food-grade carrageenan, representing κ-, ι- and λ-carrageenan, a weight-average molecular weight of 453–652 kDa and detected no obvious peak of poligeenan with a detection limit for poligeenan of about 5%;
- the ADME database was sufficient to conclude that high molecular weight carrageenan (e.g. κ/λ-carrageenan with a number-average molecular weight of approx. 800 kDa, study in rhesus monkeys) was not absorbed intact, while low molecular weight carrageenan (number-average molecular weight: 88 kDa or less) was found in tissues of rats or guinea pigs after administration of this material by gavage or diet;
- in one subchronic toxicity GLP study in rats performed with κ-carrageenan with an average molecular weight range of 196–257 kDa [not specified if number- or weight-average molecular weight; LMT of 1.9–12% < 50 kDa (mean 7%)], almost complying with the EU specification,

the NOAEL was equal to 3,394–3,867 mg/kg bw per day for males and females, respectively, the highest dose tested;

- no adverse effects have been detected in several chronic toxicity studies in rats with carrageenan (mostly κ/λ-type, no adequate indication of molecular weight distribution); from the available rat studies, NOAELs up to 7,500 mg/kg bw per day, the highest dose tested, were identified (Nilson and Wagner, 1959). However, in another study in rats given carrageenan preparations with a molecular weight of 244 and 252 kDa, a LOAEL of 1% in the diet (equivalent to 500 mg/kg bw per day) was identified by the Panel (Documentation provided to EFSA n. 43). The Panel noted that the characterisation of the test material in all the chronic studies was limited;
- carrageenan (different types) and processed Eucheuma seaweed did not raise a concern with respect to genotoxicity;
- there was no concern with respect to carcinogenicity for carrageenan (mostly κ/λ-type);
- the NOAEL of sodium and calcium κ/λ-carrageenan for developmental effects found in dietary prenatal developmental toxicity studies was 3,000 and 5,000 mg/kg bw per day for rats and hamsters (Documentation provided to EFSA n. 46), and 3,060 mg calcium carrageenan/kg bw per day for rats (Collins et al., 1977a), the highest doses tested;
- the Panel considered that for the safety evaluation of processed Eucheuma seaweed (E 407a) read across can be made from study results used in the toxicological evaluation of carrageenan (E 407).

In addition, the Panel observed that:

- from all the data received, data were adequate for a refined exposure assessment for 41 out of 79 food categories;
- in the general population, based on the reported use levels, a refined exposure, in the brand-loyal scenario of up to 758.6 mg/kg bw per day for toddlers (from 12 months up to and including 35 months of age) was estimated;
- for populations consuming foods for special medical purposes and special formulae, the 95th percentile of maximum exposure assessments calculated based on the maximum reported data from food industry (equal to the MPL) were up to 49.4 mg/kg bw per day for infants (from 12 weeks up to and including 11 months of age).

For the food additives E 407 and E 407a, the fraction of low molecular weight carrageenan is limited in the EC specifications by the purity criteria. The Panel was informed by one interested party that the material on the market does not necessarily comply with the EU specifications regarding the limit of the low molecular weight fraction. This fraction has been associated with potential health hazards similar to those reported for preparations of degraded carrageenan, such as poligeenan or C16, to which this fraction shows similarity in molecular structure and in weight-average molecular weight. Although, full identity of degraded carrageenan such as poligeenan or C16 with the low molecular weight fraction of carrageenan has not been specifically demonstrated.

Concerning degraded carrageenan (e.g. poligeenan, C16) the Panel therefore noted the following:

- degraded carrageenan has been described to be absorbed and to be present in various tissues, namely the liver, and the urine of animals when administered in drinking water or via the diet;
- degraded carrageenan did not raise concern with respect to genotoxicity;
- rats exposed to degraded ι-carrageenan (weight-average-molecular weight of 20–40 kDa) via drinking water, diet or by gavage for up to 24 months developed in first instance colitis, secondary metaplasia and finally tumours (squamous cell carcinomas, adenocarcinomas, adenomas);
- monkeys given degraded ι-carrageenan (C16) via drinking water showed histopathological lesions in the colon which varied from slight mucosal erosions at the low dose (750 mg/kg bw per day) to ulceration associated with inflammatory infiltration of the lamina propria at the high dose (2,900 mg/kg bw per day). In this study, all monkeys on C16 lost blood frequently from the intestinal tract in a dose-related degree and developed some degree of anaemia. A LOAEL of 750 mg degraded ι-carrageenan (C16)/kg bw per day was noted;
- toxicity studies have been mainly conducted with degraded ι-carrageenan, thus, degraded κ- and λ-carrageenan being sparsely toxicologically characterised.

The following uncertainties were noted by the Panel as regards the chemistry and fate of carrageenan (E 407) and processed Eucheuma seaweed (E 407a):

- no data are available showing the molecular weight distribution for different food-grade carrageenan preparations within the defined weight-average molecular weight range of 200–800 kDa;
- no data are available showing the molecular weight distribution for individual food-grade processed Eucheuma seaweed preparations;
- the weight-average molecular weight range of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) is not defined in the EU specifications allowing for the presence of a low weight-average molecular weight fraction of carrageenan. Based on the information provided on weight-average molecular weights of commercially available carrageenans, the low molecular weight material needs to be accurately quantified;
- in most of the toxicological studies the carrageenan used is not well specified and its weight-average molecular weight and its content of low molecular weight fragments are not given;
- although it has been claimed that there is no adequate analytical method available to measure the low molecular weight fraction, the Panel noted that there is indication of the percentage of the low molecular weight fraction in the food additive carrageenan (E 407) tested in a few toxicological studies;
- only limited information on the stability of carrageenan in food was available. No data on stability of carrageenan and/or processed Eucheuma seaweed addressing the usual variation of parameters (temperature, pH) relevant for the authorised food uses were available. Information on possible degradation products under acidic conditions in relevant food products is missing;
- studies investigating the hydrolysis of the κ-, ι- and λ- carrageenan showed contradictory results.

The Panel further noted the following uncertainty as regards the exposure assessment scenario:

- the refined estimates were based on 41 out of 79 food categories in which carrageenan (E 407) and processed Eucheuma seaweed (E 407a) are authorised and result in an overestimation of the real exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives in European countries considering only food additives uses for which data have been provided were considered.

Among the uncertainties from the biological and toxicological data, the Panel considered the following:

- the lack of reliable comparative toxicokinetic and toxicological studies between the different types of carrageenan and their corresponding low molecular weight fractions;
- the theoretical possibility that limited degradation could occur under conditions representative of the *in vivo* situation;
- no firm conclusion on the other types of carrageenan could be drawn on the observation of occult blood in faeces of rhesus monkeys dosed with a commercial carrageenan;
- the characterisation of the test material in most of the toxicological studies was limited;
- there were no adequate toxicological studies performed with low weight-average molecular weight carrageenan (around 200 kDa), apart from one 90-day study (with an average molecular weight carrageenan in the range of 196–257 kDa, not specified if it was a number-average or a weight-average);
- testing for chronic toxicity and reproductive and developmental toxicity was performed almost exclusively with κ/λ-carrageenan; almost no data on ι-carrageenan was available;
- inadequate data on the possible relevance of carrageenan exposure for existing inflammatory bowel diseases in humans;
- the relevance for humans of the observations in animal studies pointing to the induction of glucose intolerance and glucosuria by carrageenan is unclear;
- the possible role of sulfate and the interactions of the various forms of carrageenans with the gut microflora in some of the reported inflammatory effects of carrageenans.

Altogether in most toxicological studies, the molecular weight distribution of the carrageenan tested is not or only inadequately described. The low molecular weight fraction of carrageenan is associated with potential adverse effects. This is due to its similarity in molecular structure and weight-average molecular weight with those of degraded carrageenan, such as poligeenan or C16, known to cause inflammatory intestinal effects. Moreover, results suggesting that carrageenan might enhance inflammatory bowel disease in humans need clarification. The test compounds used in a large number of the toxicity tests are thus not considered to reflect adequately the diversity of preparations of the

food additive on the market, particularly with respect to the broad range of weight-average molecular weights reported. The Panel noted that most of these issues were also raised and discussed in a recent review (David et al., 2018).

The Panel also considered other pending uncertainties regarding the relevance of the studies available to assess the safety of the authorised food additive carrageenan (E 407). The physicochemical properties of carrageenan depend on the chemical conformation (helical or random coil) in which it exists in the preparations and in foods (solid or liquids foods) and are influenced by the presence of cations, proteins and the pH and temperature. These conditions could affect the toxicity of carrageenan and are thus relevant for the safety assessment of the food additive in the authorised uses. Furthermore, findings in some studies suggest that carrageenan exposure could be related to the induction of glucose intolerance and glucosuria. In the view of the Panel, all this information still needs clarification.

Overall, taking into account the lack of adequate data to address the above mentioned uncertainties, the Panel concluded that the existing group ADI for carrageenan (E 407) and processed Eucheuma seaweed (E 407a) of 75 mg/kg bw per day should be considered temporary, whilst the database should be improved within 5 years after publication of this opinion. Within the given time frame, high importance should be ascribed to the establishment of an adequate interlaboratory validated analytical method to quantify, at the existing 5% limit, the low weight-average molecular weight fraction of carrageenan, and to establish whether or not this fraction is associated with health risks.

The Panel further concluded that in the refined brand-loyal exposure assessment scenario the exposure estimates exceeded, in some cases by up to approximately 10-fold, the temporary existing ADI at the high level (95th percentile) for all population groups and at the mean for all population groups except for infants and the elderly. Although the exposure may be overestimated the extent of the exceedance of the ADI (10-fold) may be a safety concern.

5. Recommendations

The Panel recommended that the EC considers:

- in the definition for the food additives E 407 and E 407a in the Commission Regulation (EU) No 231/2012, specifying the weight-average molecular weight range in a narrow way avoiding a significant overlap with the molecular weight range of preparations of degraded carrageenan;
- with respect to the purity criteria in the Commission Regulation (EU) No 231/2012, the need of an interlaboratory validated analytical method to detect low molecular weight carrageenan in the food additives E 407 and E 407a at the limit specified in the Regulation;
- possibility of extending the molecular weight of the existing 5% limit for low molecular weight carrageenan from 50 kDa to up to 200 kDa for the food additives E 407 and E 407a in the Commission Regulation (EU) No 231/2012. This would take into account the limitation of the available analytical methods to quantify the percentage of low weight-average molecular weight carrageenan and will further make certain that the presence of low weight-average molecular weight carrageenan is at a low level; it should be indicated if the 5% limit refers to the food additive as such or on dried basis;
- the need of information on the stability of the food additives E 407 and E 407a in food. No data on stability of carrageenan (E 407) and/or processed Eucheuma seaweed (E 407a) addressing the usual variation of parameters (temperature, pH) relevant for the authorised food uses were available. Information on possible degradation products under acidic conditions in relevant food products was missing;
- revising the maximum limits for the impurities of toxic elements (lead, mercury, cadmium and arsenic) in the EC specification for carrageenan (E 407) and processed Eucheuma seaweed (E 407a) in order to ensure that the food additives will not be a significant source of exposure to these toxic elements in food;
- collecting monitoring data for the food categories contributing the most to the exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) in order to allow for more refined estimates of exposure;
- establishing, as a general principle, numerical MPLs for all food authorised uses with special consideration of the main food categories contributing to the exposure (e.g. flavoured milk products, flavoured drinks, food supplements and fine bakery wares) to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives in order to reduce consumers exposure.

Documentation provided to EFSA

1) Pre-evaluation documents on carrageenan (E 407). Frauenhofer ITEM. December 2012.
2) Pre-evaluation documents on processed Eucheuma seaweed (E 407a). Frauenhofer ITEM. December 2012.
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12) Marinalg International, 2015. Reply to EFSA: call for technical data on certain thickening agents permitted as food additives in the EU. Information on carrageenan (E 407) on: overview document "New and significant published studies and information critical to the EFSA re-evaluation of the safety of carrageenan for use as a food additive"; Appendix A and B, "Carrageenan studies published since 8 November, 2010 submission to EFSA re-evaluation" (spreadsheet and copies of the papers); Appendix C, "Status report on the work of Marinalg International to measure the molecular weight distribution of carrageenan and processed Eucheuma seaweed in order to meet the EU specification: less than 5% below 50 kDa; Submitted on 26 November 2015.
13) Marinalg International, 2016. Reply to EFSA. Additional studies published McKim et al. (2016), "*Effects of carrageenan on cell permeability, cytotoxicity, and cytokine gene expression in human intestinal and hepatic cell lines*". Submitted on 22 September 2016.
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carrageenan (E 407a) on and processed Eucheuma seaweed (E 407a) on identity, specifications and manufacturing process, and a report on the measurement of the molecular weight distribution of carrageenan and processed Eucheuma seaweed. Submitted on 22 December 2015.
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20) AESGP (Association of the European Self-Medication Industry), 2014. Data on usage levels on carrageenan (E 407) and processed Eucheuma seaweed (E 407a) in response to the EFSA call for food additives usage level and/or concentration data in food and beverages intended for human consumption (Batch 3). Submitted to EFSA by 30 September 2013.
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Glossary and Abbreviations

AAS	atomic absorption spectroscopy
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and excretion
AESGP	Association of the European Self-Medication Industry
AFC	Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
ALT	alanine transaminase
ANS	Panel on Food Additives and Nutrient Sources added to Food

AOAC	Association of Analytical Communities
AOM	azoxymethane
ARC	alternatively refined carrageenan
ASEMESA	Asociacion Espanola de Exportadores e Industriales de Aceitunas de Mesa
AST	aspartate transaminase
BCL 10	B-cell lymphoma/leukaemia 10
CAS	Chemical Abstracts Service
CFU	colony forming unit
CHL	Chinese hamster lung fibroblast
CLBG	clarified locust bean gum
DEAE	diethylaminoethyl
DMH	dimethyhydrazine
DNPH	2,4-dinitrophenylhydrazine
EC	Evaluated under Commission
EDA	European Dairy Association
EINECS	European Inventory of Existing Commercial Chemical Substances
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FAO/WHO	Food and Agriculture Organization/World Health Organisation
FC	Food for weight control
FCS	Food Classification System
FDA	Food and Drug Administration
FDE	FoodDrinkEurope
FDRL	Food and Drug Research Laboratories
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
FSMP	Foods for special medical purposes
GC	gas chromatography
GD	gestational day
GERD	gastroesophageal reflux disease
GI	gastrointestinal
GLP	good laboratory practice
GNPD	Global New Products Database
GPC/ICP	gel permeation/inductively coupled plasma
GTT	glucose tolerance test
HMPC	Committee on Herbal Medicinal Products
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HPSEC-RI	high-performance size exclusion chromatography coupled to a refractive index detector
IARC	International Agency for Research on Cancer
ICGA	International Chewing Gum Association
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
INS	International Numbering System for Food Additives
IOM	Institute of Medicine
IR	infrared
ITT	insulin resistance test
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC–MS/MS	liquid chromatography with tandem mass spectrometry
LD_{50}	lethal dose, 50% i.e. dose that causes death among 50% of treated animals
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
LMT	low molecular weight tail
LS	light scattering
MALS	Multiple Angle
MoE	margins of exposure

MPL	maximum permissible level
NDA	Panel on Dietetic Products, Nutrition and Allergies
NF-κB	nuclear factor κB
NOAEL	no-observed-adverse-effect level
OECD	Organisation for Economic Co-operation and Development
PES	processed Eucheuma seaweed
PND	postnatal day
PNG	Philippine natural grade
QS	*quantum satis*
ROS	reactive oxygen species
RSD	relative standard deviation
SA	sodium alginate
SCE	sister chromatid exchange
SCF	Scientific Committee for Food
SCFA	short-chain fatty acids
SEC	size exclusion chromatography
SGPT	serum glutamic pyruvic transaminase
SIDS	Screening Information Dataset
SmPC	Summary of product characteristics
SNE	Specialised Nutrition Europe
SRC	semi-refined carrageenan
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-α
TNO	Netherlands Organisation for Applied Scientific Research
UDS	unscheduled DNA synthesis
USAN	United States Adopted Names
UV	ultraviolet
WHO	World Health Organisation

Appendix A – Summary of reported use levels (mg/kg or mg/L as appropriate) of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) provided by industry

Appendix B – Summary of reported use levels (mg/kg or mg/L as appropriate) of processed Eucheuma seaweed (E 407a) provided by industry

Appendix C – Summary of analytical results (mg/kg or mg/L as appropriate) of carrageenan (E 407) provided by Member States

Appendix D – Number and percentage of food products labelled with carrageenan (E 407) and processed Eucheuma seaweed (E 407a) out of the total number of food products present in the Mintel GNPD per food subcategory between 2012 and 2017

Appendix E – Concentration levels of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) used in the exposure assessment scenarios (mg/kg or mL/kg as appropriate)

Appendix F – Summary of total estimated exposure of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) from their use as food additives for the maximum level exposure scenario and the refined exposure assessment scenarios per population group and survey: mean and 95th percentile (mg/kg bw per day)

Appendix G – Main food categories contributing to exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) using the maximum level exposure assessment scenario and the refined exposure assessment scenarios (> 5% to the total mean exposure)

Appendix H – Summary of anticipated exposure to carrageenan (E 407) and processed Eucheuma seaweed (E 407a) taking into account the consumption of food supplements, for consumers only, among children, adolescents, adults and the elderly (minimum–maximum across the dietary surveys in mg/kg bw per day)

Appendix I – Sources for the production of carrageenan

Over the last few years, most of the seaweeds used and authorised according to the EU specification for the production of carrageenan have been reclassified by marine biologists as they gain more knowledge of their structure. If readers are to go beyond this publication for further information, they need to be familiar with the both the old and new names, as listed below:

- *Betaphycus gelatinum* was *Eucheuma gelatinae*.
- *Chondrus crispus* remains unchanged, and is commonly known as 'Irish moss'.
- *Eucheuma cottonii* is now *Kappaphycus alvarezii*, and commercially was and is called 'cottonii'.
- *Eucheuma denticulatum* was *Eucheuma spinosum* and commercially was and is called 'spinosum'.
- *Eucheuma gelatinae* is now *Betaphycus gelatinum*.
- *Eucheuma spinosum* is now *Eucheuma denticulatum* and commercially was and is called '*spinosum*'.
- *Gigartina canaliculata* remains unchanged.
- *Gigartina skottsbergii* remains unchanged.
- *Gigartina stellata*, mentioned in earlier articles but now not so important, is now *Mastocarpus stellatus.*
- *Hypnea musciformis* remains unchanged.
- *Iridaea ciliata* is now *Sarcothalia crispata*.
- *Iridaea laminaroides* is now *Mazzaella laminaroides*.
- *Kappaphycus alvarezii* was *Eucheuma cottonii* and commercially was and is called '*cottonii*'.
- *Mazzaella laminaroides* was *Iridaea laminaroides*.
- *Mastocarpus stellatus* was *Gigartina stellata*, mentioned in earlier articles but now not so important.
- *Sarcothalia crispata* was *Iridaea ciliata*.

Appendix J – Interpretation of the identity of samples used in ADME and Toxicity investigations

Introductory text from Section 3.5

The investigations used in the present opinion to disclose the ADME as well as the toxicity are spread over a time period from 1941 and to the present day. In this connection, the Panel noted, that:

- The sources of carrageenan have changed over time as already discussed in Section 3.1.1.1.
- The original source was *Chondrus crispus* which still is used.
- Today most carrageenan, however, is coming from the two algae *Eucheuma cottonii* and *Eucheuma spinosum*.
- Each biological source presents a unique composition of the three-known carrageenans.
- ι and κ (most often in natural mixtures) so far have been the most used carrageenans.
- Pure λ-carrageenan recently has become more used in the US (TCI America, Portland, OR, USA (at: http://www.tcichemicals.com/eshop/en/us/commodity/C3313/) and Modernist Pantry, Eliot, ME, USA (at: http://www.modernistpantry.com/carrageenan-λ.html).
- The way of describing samples used in investigations has changed during the long period of time mentioned.

Thus, taking the time as well as the source aspect into consideration, the Panel decided that in order to make it possible to compare the results of different studies, an interpretation of the identity (expressed in type(s) of carrageenan(s)) should be made for each sample.

General about the possibilities for, and methods to, identify a sample to an overall composition with regard to the three carrageenans, as maybe added information about the overall molecular size (mean and molecular weight distribution).

1. Each carrageenan used may only be characterised based on the information given in the publication (incl. appendices etc.).

   1.1. This information may include:

      1.1.1. Producer
      1.1.2. Commercial Brand name including information on subqualities (if any)
      1.1.3. Producers commodity (part) number
      1.1.4. Purity – regarding the occurrence of non-carrageenan compounds of organic and/or inorganic nature
      1.1.5. Biological origin (Algal species)
      1.1.6. Geographical origin
      1.1.7. Type of carrageenan according to the in general accepted subdivision into the types of ι-, κ- and λ-carrageenans. If given, this may be as one of the three types or as a mixture, with or without percentages of occurrence of each type.
      1.1.8. Molecular weight: as a number-average and/or as a weight-average molecular weight
      1.1.9. Molecular weight distribution (in weight percentage. In case the product is stated to be a mixture of two or three of the above-mentioned types of carrageenan, information about molecular weight (average and distribution) may be given separately for each type.
      1.1.10. Content if any of degraded carrageenan, their origin and characteristics

2. Working with older literature, most often many of the above pieces of information are lacking. However, in some cases, it is possible to deduce further information from those already given.

   2.1. An example would be the deduction of the type(s) of carrageenan(s) in a product for which the biological origin (algal species) is given.

      2.1.1. In this latter case, it should be noted, that the species may have changed name. Important name changes are summarised in Annex A.

3. Writing the present Opinion 'Re-evaluation of carrageenan (E 407) and processed Eucheuma seaweed (E 407a) as food additives', the Panel as already decided to use all existing possibilities for deduction of information – further to that already directly provided - about the products used in the experiments incorporated into the Opinion. Thus, for each

experiment incorporated one will find a paragraph summarising The information provided and the interpretation of this, concerning the carrageenan used.

3.1. This above-mentioned paragraph will occur at the end of the text describing the experiment/test in question, and will present itself as follows: Carrageenan used (1) information provided: xxx (2) Interpretation of the information: xxx

I U C L I D

D a t a s e t

Existing Chemical	Substance ID: 9000–07–1
CAS No.	9000–07–1
EINECS Name	Carrageenan
EINECS No.	232–524–2
Molecular Formula	<no data>

Dataset created by: EUROPEAN COMMISSION – European Chemicals Bureau

This dossier is a compilation based on data reported by the European Chemicals Industry following 'Council Regulation (EEC) No. 793/93 on the Evaluation and Control of the Risks of Existing Substances'. All (non–confidential) information from the single datasets, submitted in the IUCLID/HEDSET format by individual companies, was integrated to create this document.

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European Chemicals Bureau

1.0.1 OECD and Company Information

Name: Danisco Ingredients
Street: Edwin Rahrs Vej 38
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Telefax: +45 86 25 10 77
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Name: FMC Corporation
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Country: Netherlands
Phone: xx–31–70–3150000
Telefax: xx–31–70–3989893
Telex: 30909

Name: Quest International
Street: Kilnagleary, Carrigaline
Town: Co. Cork
Country: Ireland
Phone: (21)372541
Telefax: (21)372652
Telex: 75071

1.0.2 Location of Production Site

–

1.0.3 Identity of Recipients

–

1.1 General Substance Information

Substance type: natural substance
Physical status: solid

1.1.1 Spectra

–

1.2 Synonyms

Carrageen
Source: Danisco Ingredients Brabrand

Carrageenin
Source: FMC Corporation Manchester
Danisco Ingredients Brabrand

Carrageenin; E407; Euchema spp extract, Danish agar, Irish moss extract
Source: Quest International Co. Cork

E 407
Source: FMC Corporation Manchester

E407
Source: Hercules BV Rijswijk

Eucheuman (Eucheuma spp.) (proposed)
Source: FMC Corporation Manchester

Furcellaran or Danish agar (from Furcellaria fastigiata) (proposed)
Source: FMC Corporation Manchester

Hypnean (from Hypnea spp.) (proposed)
Source: FMC Corporation Manchester

Iridophycan (Irdidaea spp.) (proposed)
Source: FMC Corporation Manchester

Irish Moss Extract (proposed)
Source: FMC Corporation Manchester

Irish moss gelose (proposed)
Source: FMC Corporation Manchester

1.3 Impurities

-

1.4 Additives

-

1.5 Quantity

-

1.6.1 Labelling

-

1.6.2 Classification

-

1.7 Use Pattern

Type: type
Category: Non dispersive use

Type: type
Category: Use resulting in inclusion into or onto matrix

Type: type
Category: Wide dispersive use

Type: industrial
Category: other: food industry

Type: industrial
Category: other

Type: use
Category: Food/foodstuff additives

Type: use
Category: Stabilizers

Type: use
Category: other: Pharmaceuticals: excipient

Type: use
Category: other: food additives, emulsifier, gelling agent, dispersant, stabilizer

1.7.1 Technology Production/Use

-

1.8 Occupational Exposure Limit Values

Type of limit: other
Limit value:
Remark: None established.
Source: Quest International Co. Cork

Type of limit: other: None available. Wear respirators which are approved by NIOSH/MSHA (or equivalent agency) for protection against airborne dust when it cannot be controlled.
Limit value:
Source: FMC Corporation Manchester

Type of limit:
Limit value:
Remark: Non available.
Source: Hercules BV Rijswijk

1.9 Source of Exposure

Remark: Not applicable (food additive).
Source: Hercules BV Rijswijk

Remark: Not applicable: Food Additive.
Source: FMC Corporation Manchester

Remark: Not applicable, material is an approved food additive.
Source: Quest International Co. Cork

1.10.1 Recommendations/Precautionary Measures

-

1.10.2 Emergency Measures

-

1.11 Packaging

-

1.12 Possib. of Rendering Subst. Harmless

-

1.13 Statements Concerning Waste

-

1.14.1 Water Pollution

Classified by: other: not applicable
Labelled by:
Class of danger:
Source: FMC Corporation Manchester

1.14.2 Major Accident Hazards

-

1.14.3 Air Pollution

-

1.15 Additional Remarks

Remark: The Chapters 2-5 have been filled out by FMC Corporation.
Source: Hercules BV Rijswijk

Remark: Carrageenan (E 407) has been an approved food additive for more than 50 years worldwide. In the EU, carrageenan is listed in Annex I at Quantum Satis (QS) in the European Parliament and Council Directive No. 95/2/EC of 20 February 1995 on "Food Additives Other Than Colours and Sweeteners". Carrageenan is used primarily as a gelling agent and fat substitute in concentrations of 0.1-0.5% in various foodstuffs. Carrageenan is naturally occurring, extracted from certain seaweed species. Carrageenan is produced according to approved manufacturing processes and complies with the identity and specifications criteria established in the European Community, as per the Council Directive 8/663/EEC of 25 July 1978, as amended, "Laying Down Specific Criteria of Purity for Emulsifiers, Stabilizers, Thickeners and Gelling Agents for Use in Foodstuffs". Carrageenan has been toxicologically evaluated by national authorities in the EU and the EU Scientific Committee for Food (SCF). The Acceptable Daily Intake (ADI) currently established in the EU is 0-75 mg/kg bodyweight.
Source: FMC Corporation Manchester

Remark: None
Source: Quest International Co. Cork

1.16 Last Literature Search

-

1.17 Reviews

-

1.18 Listings e.g. Chemical Inventories

-

2.1 Melting Point

Value:
Remark: Not applicable
Source: FMC Corporation Manchester

2.2 Boiling Point

Value:
Remark: Not applicable
Source: FMC Corporation Manchester

2.3 Density

Type: bulk density
Value:
Remark: See Chapter 2.12.
Source: FMC Corporation Manchester

2.3.1 Granulometry

–

2.4 Vapour Pressure

Value:
Remark: Not applicable
Source: FMC Corporation Manchester

2.5 Partition Coefficient

log Pow:
Method:
Year:
Remark: Not applicable
Source: FMC Corporation Manchester

2.6.1 Water Solubility

Remark: See Chapter 2.12.
Source: FMC Corporation Manchester

2.6.2 Surface Tension

–

2.7 Flash Point

Value:
Type:
Method:
Year:
Remark: Not applicable
Source: FMC Corporation Manchester

2.8 Auto Flammability

Value:
Remark: Not applicable
Source: FMC Corporation Manchester

2.9 Flammability

Result:
Remark: Not applicable
Source: FMC Corporation Manchester

2.10 Explosive Properties

Result: no data
Remark: Accumulation of overhead settled dust may form explosive concentrations in air when disturbed and dispersed.
Source: FMC Corporation Manchester

2.11 Oxidizing Properties

Result: no oxidizing properties
Source: FMC Corporation Manchester

2.12 Additional Remarks

Remark: Carrageenan E 407 is a naturally occurring substance extracted from certain seaweed species including Furcellaria, Chondrus, Hypnea, Iridaea, and Eucheuma. Carrageenan is produced according to approved manufacturing processes and complies with the identity and purity criteria established in the European Community. See Section 1.13.
Source: FMC Corporation Manchester

3.1.1 Photodegradation

Type: other: See Chapter 3.8.
Method:
Year: **GLP:**
Test substance:
Source: FMC Corporation Manchester

3.1.2 Stability in Water

Type:
Method:
Year: **GLP:**
Test substance:
Remark: See Chapter 3.8.
Source: FMC Corporation Manchester

3.1.3 Stability in Soil

Type: other: See Chapter 3.8. **Radiolabel:**
Concentration:
Cation exch. capac.
Microbial biomass:
Method:
Year: **GLP:**
Test substance:
Source: FMC Corporation Manchester

3.2 Monitoring Data (Environment)

Type of measurement: other: See Chapter 3.8.
Medium:
Source: FMC Corporation Manchester

3.3.1 Transport between Environmental Compartments

Type:
Media:
Method:
Year:
Remark: See Chapter 3.8.
Source: FMC Corporation Manchester

3.3.2 Distribution

Media: other: See Chapter 3.8.
Method:
Year:
Source: FMC Corporation Manchester

3.4 Mode of Degradation in Actual Use

Remark: Will not degrade in actual use.
Source: FMC Corporation Manchester

3.5 Biodegradation

Type:
Inoculum:
Method:
Year: **GLP:**
Test substance:
Remark: See Chapter 3.8.
Source: FMC Corporation Manchester

3.6 BOD5, COD or BOD5/COD Ratio

Method: other

Remark: See Chapter 3.8.
Source: FMC Corporation Manchester

3.7 Bioaccumulation

Species:
Exposure period:
Concentration:
BCF:
Elimination:
Method:
Year: **GLP:**
Test substance:
Remark: See Chapter 3.8.
Source: FMC Corporation Manchester

3.8 Additional Remarks

Remark: Carrageenan is extracted from a natural source (seaweed) and consequently would not be expected to have any significant negative effect on the environment.
Source: FMC Corporation Manchester

AQUATIC ORGANISMS

4.1 Acute/Prolonged Toxicity to Fish

Type: static
Species: Lepomis macrochirus (Fish, fresh water)
Exposure period: 96 hour(s)
Unit: mg/l **Analytical monitoring:** no
NOEC: = 1
Method:
Year: 1974 **GLP:** no
Test substance: other TS: Hercules HMR Carrageenan
Source: FMC Corporation Manchester

(1)

4.2 Acute Toxicity to Aquatic Invertebrates

-

4.3 Toxicity to Aquatic Plants e.g. Algae

-

4.4 Toxicity to Microorganisms e.g. Bacteria

-

4.5 Chronic Toxicity to Aquatic Organisms

4.5.1 Chronic Toxicity to Fish

-

4.5.2 Chronic Toxicity to Aquatic Invertebrates

-

TERRESTRIAL ORGANISMS

4.6.1 Toxicity to Soil Dwelling Organisms

–

4.6.2 Toxicity to Terrestrial Plants

–

4.6.3 Toxicity to other Non–Mamm. Terrestrial Species

–

4.7 Biological Effects Monitoring

–

4.8 Biotransformation and Kinetics

–

4.9 Additional Remarks

Remark: Carrageenan is a an extract from Eucheuma, Chondrus, Iridaea and Hypnea seaweed and is not expected to have significant toxicity to aquatic and other organisms.

Source: FMC Corporation Manchester

5.1 Acute Toxicity

5.1.1 Acute Oral Toxicity

Type: LD50
Species: rat
Sex:
Number of Animals:
Vehicle:
Value: = 5400 mg/kg bw
Method: other: Five rats/sex were administered the test substance once at dosage levels of 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 g/kg and observed for 14 days for signs of toxicity. Dosing was by gavage.
Year: 1971 **GLP:** no
Test substance: other TS: Calcium carrageenan
Remark: Results: Mortality at the dosage levels was: 0 (3.0), 10% (4.0), 10% (5.0), 70% (6.0), 90% (7.0), 100% (8.0 g/kg). signs of toxicity included uneasiness, increased body temperature, anorexia, muscle tremors, prostration, and death. The results indicate that on acute oral exposure the test substance was practically nontoxic.
Source: FMC Corporation Manchester

(2)

Type: LD0
Species: rat
Sex:
Number of Animals:
Vehicle:
Value: > 5000 mg/kg bw
Method: other: US EPA Toxic Substances Health Effects Test Guidelines Acute Oral Toxicity
Year: 1983 **GLP:** yes
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Five rats/sex were administered a 25% (w/v) slurry of the test material in corn oil at a dosage level of 5000 mg/kg, once by gavage. They were then observed for 14 days for signs of toxicity. A necropsy was performed on all rats at study termination.

Results: No animals died during the course of the study. There were no signs of toxicity observed during the in-life portion or at necropsy. These results indicate that the test material is practically nontoxic when administered in a single dose orally.
Source: FMC Corporation Manchester

(3)

Type: LD50
Species: mouse
Sex:
Number of Animals:
Vehicle:
Value: = 9150 mg/kg bw
Method: other: Five mice/sex were administered the test material as a 25% corn oil suspension once at dosage levels of 5.0, 7.0, 8.0, 10, and 12 g/kg and animals were observed for signs of toxicity for 14 days. Administration was by gavage.
Year: 1971 **GLP:** no
Test substance: other TS: Calcium carrageenan
Remark: Results: Percent mortality observed per group was 0 (5.0 g/kg), 10% (7 g/kg), 10% (8 g/kg), 70% (10.0 g/kg) and 90% (12.0 g/kg). Clinical signs of toxicity included uneasiness, anorexia, muscle tremors, soft stools, prostration and death. The results indicate that on acute oral exposure the test substance was practically nontoxic.
Source: FMC Corporation Manchester

(4)

Type: LD50
Species: rabbit
Sex:
Number of Animals:
Vehicle:
Value: = 2640 mg/kg bw
Method: other: Five rabbits/sex were administered the test article suspended in corn oil (25%) by gavage at dosage levels of 1.0, 2.0, 3.0, 4.0 and 5.0 g/kg and they were observed for 14 days for signs of toxicity.
Year: 1971 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Results: The deaths in each dosage group were 0 (1.0 g/kg), 30% (2.0 g/kg), 60% (3.0 g/kg), 70% (4.0 g/kg), 100% (5.0 g/kg). Signs of toxicity included malaise, increased body temperature, anorexia, muscular twitching, prostration.
Source: FMC Corporation Manchester

(5)

Type: LD50
Species: hamster
Sex:
Number of Animals:
Vehicle:
Value: = 6750 mg/kg bw
Method: other: Five hamsters/sex were administered the test substance as a 25% corn oil suspension by gavage once at dosage levels of 4.0, 5.0, 6.0, 7.0, and 8.0 g/kg. Animals were then observed for signs of toxicity for 14 days.
Year: 1971 **GLP:** no
Test substance: other TS: calcium carrageenan
Remark: Results: Deaths in the various dosage groups were: 20% (4.0 g/kg), 10% (5.0 g/kg), 40% (6.0 g/kg), 50% (7.0 g/kg)

and 70% (8.0 g/kg). Signs of toxicity included malaise, anorexia, muscle spasms, soft stools, prostration. The results indicate that on acute oral exposure the test substance was practically nontoxic.

Source: FMC Corporation Manchester

(6)

5.1.2 Acute Inhalation Toxicity

Type: LC0
Species: rat
Sex:
Number of Animals:
Vehicle:
Exposure time: 4 hour(s)
Value: = .93 mg/l
Method:
Year: 1983 **GLP:** yes
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: Two groups containing 5 rats/sex each were exposed to either the test compound at the highest attainable dust concentration, or HEPA-filtered room air (control) for 4 hours and then were examined for signs of toxicity for 14 days. All animals received full necropsies at termination. Chamber air was sampled and analyzed for test substance concentration at 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 hours during exposure. The particle size distribution was measured at 1.0 and 3.0 hours into the exposure period.

Results: The mean air concentration over the 4 hour test period was 930.8 mg/m3 (converted to 0.93 mg/l, highest attainable dust concentration) and the particle size was 8.3-8.8 uM. There were no deaths in any rat during the course of the study and the only signs of toxicity in the treated group were conjunctivitis and lacrimation which cleared by day 7 following exposure.

Source: FMC Corporation Manchester

(7)

5.1.3 Acute Dermal Toxicity

Type: LD0
Species: rabbit
Sex:
Number of Animals:
Vehicle:
Value: = 2000 mg/kg bw
Method: other: US EPA Toxic Substances Health Effects Test Guidelines Acute Dermal Toxicity
Year: 1983 **GLP:** yes
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: The trunks of 5 rabbits/sex were clipped free of hair on the day prior to test material administration. Test

sites consisting of about 10% of the animal's body surface were abraded just prior to dosing. A dosage level of 2000 mg/kg was tested. It was weighed onto a 4 x 4 inch gauze pad and moistened with physiological saline. The test site was occluded with impervious plastic sheeting for 24 hours. A collar was used to keep the animal from ingesting the material. Animals were observed for signs of toxicity for 14 days after dosing. Skin scores were recorded on day 1, 3, 7, and 14 of study. Rabbits received a full necropsy at study termination.

Results: No deaths occurred in any of the rabbits during the course of the study. The LD50 was considered to be greater than 2000 mg/kg in both male and female rabbits.

Source: FMC Corporation Manchester

(8)

5.1.4 Acute Toxicity, other Routes

–

5.2 Corrosiveness and Irritation

5.2.1 Skin Irritation

Species: rabbit
Concentration:

Exposure:
Exposure Time:
Number of Animals:
PDII:
Result: not irritating
EC classificat.: not irritating
Method: other: US EPA Toxic Substances Health Effects Guidelines Primary Dermal Irritation
Year: 1983 **GLP:** yes
Test substance: as prescribed by 1.1 – 1.4
Remark: Method: Three rabbits/sex were used for this test. The trunks were clipped free of hair and two sites were identified adjacent to the spinal column on each side. The two sites on the right side were abraded and the others left intact. 0.5 g test material, moistened with physiological saline was placed in a 1 square inch pad and this was taped to each site. The entire trunk was then wrapped with impervious non–reactive plastic sheeting. 24 hours after dosing the wrappings were removed and sites were wiped with gauze. About 30 minutes later the four sites were scored using the Draize method. The sites were again evaluated at 72 hours.

Results: Two rabbits had slight erythema over the abraded test sites 24 hours after application. Within 72 hours all irritation had resolved.

Source: FMC Corporation Manchester

(9)

5.2.2 Eye Irritation

Species: rabbit
Concentration:
Dose:
Exposure Time:
Comment:
Number of Animals:
Result: not irritating
EC classificat.: not irritating
Method: other: US EPA Toxic Substances Health Effects Test Guidelines Primary Eye Irritation
Year: 1983 **GLP:** yes
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Nine rabbits were used in this study. All received 0.1 g test substance in the conjunctival pouch of the right eye. In three of the rabbits the treated eye was washed with 100 ml tap water about 20–30 seconds after instillation. Eyes were scored using the method of Draize at 1, 24, 48 and 72 hours after dosing. Fluorescein dye was used at 24 hours and thereafter in eyes testing positive at 24 hours.

Results: Slight conjunctiveal irritation was seen equally in washed and unwashed eyes at the 1 and 24 hour intervals. At the 48 hour interval no irritation was observed in any animal.
Source: FMC Corporation Manchester

(10)

5.3 Sensitization

Type: other: modified Buehler patch test
Species: guinea pig
Number of Animals:
Vehicle:
Result: not sensitizing
Classification: not sensitizing
Method: other: see Remarks
Year: 1983 **GLP:** yes
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: The study design followed the Buehler method although no mention was made to this in the report. Ten male guinea pigs were prepared by shaving and depilating the backs. They were then patched with a 0.5 ml slurry of 500 mg test substance mixed with 1.5 ml deionized water on study days 0, 2, 5, 7, 9, 12, 14, 16, 19 and 21. Adhesive patches were placed on the backs and the torso was wrapped with polyethylene film to secure the patches in place. Animals were then placed in a restrainer for 6 hours at which time the wrappings and patches were removed and they were

returned to their cages. Skin reactions were scored 24 hours after each treatment as well as at about 48 hours after treatment 1. On day 35 the animals were patched again with the same slurry for 6 hours (challenge patch) and the reaction was read at 24 and 48 hours after treatment.

A group containing 10 other male guinea pigs received a positive control material (0.05% 2,4-dinitrochlorobenzene in ethanol) according to the same procedures as the test substance.

Results: A pre-screen showed that the highest non-irritating concentration of the test material was 500 mg in 1.5 ml deionized water.

The definitive test indicated that the test material was not a sensitizer in this model. The positive control group showed a positive response to the challenge patch, as expected.

Source: FMC Corporation Manchester

(11)

5.4 Repeated Dose Toxicity

Species: rat **Sex:** male
Strain: Sprague-Dawley
Route of admin.: oral feed
Exposure period: 4 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 0, 5%, 10%, 15% of diet
Control Group: yes, concurrent no treatment
Method: other: See remark
Year: **GLP:** no data
Test substance: other TS: obtained from Sigma Co.
Remark: Methods: Four groups containing 5 male rats each were administered synthetic diets containing 0% (no fiber), 5%, 10% and 15% carrageenan. Another group received 5% cellulose. All diets contained 1% cholesterol in addition to varying amounts of corn oil. The rats were maintained on these diets for 28 days. Body weights and food consumption were measured and at necropsy blood was obtained and cholesterol level was measured. Mucinase content was measured in fecal samples.

Results: No significant differences were observed in body weight gain or total food intake across groups. Fecal mucinase activities were high in the 15 and 10% carrageenan diet groups followed by the fiber-free group and the 5% cellulose and the 5% carrageenan diet groups. The differences between the 15% carrageenan group and the other dietary groups were significant also. Activity in the 10% carrageenan diet group was significantly higher than in the 5% cellulose and 5% carrageenan groups. Rats fed any of the

fiber containing diets had similar serum cholesterol levels and they all were significantly lower than the fiber-free control group.

Conclusion: Diets containing the lower level of fiber (5% cellulose or carrageenan) showed no deleterious effect on mucinase activity, or body weight gain. Both lowered cholesterol level significantly as compared to the controls. The high levels of carrageenan employed in this study are irrelevant to potential human exposure.

Source: FMC Corporation Manchester

(12)

Species: rat **Sex:** male/female
Strain: Sprague-Dawley
Route of admin.: oral feed
Exposure period: 13 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 1%, 5%
Control Group: yes, concurrent no treatment
NOAEL: = 5 %
Method:
Year: 1985 **GLP:** no data
Test substance: other TS: Iridaea carrageenan/Hercules Inc.
Remark: Methods: Three groups containing about 15 rats/sex each were administered carrageenan in the diet at dietary levels of 0, 1, and 5% for 13 weeks. The control group received Alphacel at 5%. Animals were observed for signs of toxicity, body weights and food consumption were taken weekly, hematology and clinical chemistry parameters were measured at the end of the study, and urinalysis was performed at the same time. Select organ weights were measured and specimens of liver, kidney, cecum, colon and anorectal junction from 10 rats/sex from the control and 5% carrageenan groups were stained and examined for metachromasia. Livers were also examined under electron microscope.

Results: The only effects observed were a decrease in sodium and potassium levels and an increase in SGPT in the plasma of the treated groups at study termination. These groups also showed the presence of Councilman bodies in the livers, but the physiological significance of this finding was unknown. Carrageenan caused no significant toxicological effects when fed in the diet at 5% in rats for 13 weeks.

Source: FMC Corporation Manchester

(13)

Species: rat **Sex:** female
Strain: Wistar
Route of admin.: oral feed
Exposure period: 6–7 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 0, 10, 20, 40 % of diet
Control Group: yes, concurrent no treatment
LOAEL: = 10 %
Method:
Year: 1981 **GLP:** no data
Test substance: other TS: carrageenan
Remark: Methods: Groups of female Wistar rats were administered either a control defined diet without any fiber, or a diet containing 10%, 20%, or 40% carrageenan for 6–7 weeks. Body weights were taken every 2 days, feces and urine were collected at 2 day intervals and analyzed for volume, water content; water intake was measured daily. At the end of the feeding period the animals were sacrificed and the small intestine, cecum and colon were removed, measured and weighed. The mucosa was scraped off the small intestine evaluated for protein and DNA content.

Results: Carrageenan was found to be inert (not metabolized) in this model. Therefore the rats responded to the energy dilution of the diet by increasing food intake proportionaly at the different concentrations. Because the carrageenan used was mainly the sodium salt, the increase in fecal water excretion appeared to be overcompensated by a drastic rise in daily water intake. Increasing colon weight was observed with increased dietary fiber. Also, increasing the amount of the carrageenan in the diet resulted in significant increase in length of the small intestine. Mucosal protein and DNA content also increased as concentration of fiber in the diet increased.

As the carrageenan content of the diet increased more colonic work was needed to propel the more bulky contents of the feces along the large intestine, and this led to muscle hypertrophy.
Source: FMC Corporation Manchester

(14)

Species: rat **Sex:** male
Strain: Fischer 344
Route of admin.: oral feed
Exposure period: 28, 64 and 91 days
Frequency of treatment: continuous
Post. obs. period: 0 or 28 days
Doses: 5% iota carrageenan in diet
Control Group: yes, concurrent no treatment
Method:
Year: 1992 **GLP:** no data
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Rats were fed diets containing 0, or 5% of carrageenan for 28, 64 or 91 days. Those groups treated for 64 days were put on an untreated diet for another 28 days to evaluate reversibility of effects. Colons were removed and stained for PCNA, and tissues were examined grossly.

Results: There was no increase in PCNA–positive cells in the upper third of the crypt in the carrageenan treated group at 28 days. The carrageenan fed animals exhibited an 8–fold increase in the number of labeled cells in the upper third of the crypt after 91 days, but no PCNA–positive cells were ever observed at the luminal surface. In the group fed carrageenan for 64 days followed by a 28–day recovery period, proliferating cells returned to a location and level similar to those in the control group.
Source: FMC Corporation Manchester

(15)

Species: rat **Sex:** male/female
Strain: Sprague–Dawley
Route of admin.: oral feed
Exposure period: 13 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 0, 1% Type S carrageenan, 5% Type S, 1% Type C, 5% Type C, 1% Type AX, 5% Type AX
Control Group: yes, concurrent no treatment
Method:
Year: 1985 **GLP:** no data
Test substance: other TS: 3 carrageenans (Carastay) obtained from Stauffer Chemical Co.
Remark: Methods: In order to compare the toxicity of carrageenans with different viscosities in rats, eight groups consisting of 25 rats/sex each were fed diets containing either 5% Alphacel (control) or the following carrageenans for 13 weeks: 1% Type S (70:30 kappa:lambda, visc. 270 cps, 6% sodium); 5% Type S; 1% Type C (80:20 kappa:lambda, visc. 190 cps and 6% potassium; 5% Type C; 1% Type AX (90:10 kappa:lambda, visc 26 cps and 6% calcium); 5% Type AX. Growth and survival were monitored and at sacrifice blood was taken for hematological and chemical evaluation. A full complement of tissues from the groups receiving 5% of each

carrageenan and the controls were examined microscopically. Livers were examined for stored carrageenan using extraction procedures.

Results: No differences in mortality or growth pattern was observed between groups after 13 weeks of dosing. Clinical pathology and microscopic pathology were all comparable across groups and as compared to the controls. No extractable carrageenan or derivative was found in any of the livers examined.

Therefore there is no difference between the three types of carrageenans administered. None appear to be absorbed from the gut and stored in the liver. There were no indications of toxicity when each was fed at up to 5% in the diet for 13 weeks.

Source: FMC Corporation Manchester

(13)

Species: rat **Sex:** male
Strain: Wistar
Route of admin.: oral feed
Exposure period: 56 days
Frequency of treatment: continuous
Post. obs. period: none
Doses: 5%
Control Group: no
NOAEL: = 5 %
Method:
Year: 1973 **GLP:** no
Test substance: other TS: Glaxo native carrageenan
Remark: Methods: 10 male rats were administered carrageenan in the diet for 56 days. They were then sacrificed and the gastrointestinal tract was removed and examined histologically. Stains were used to determine the presence of carrageenan and lysosomal enzymes.

Results: The only clinical sign observed was slight diarrhea, marked chiefly by feces which were semi-solid in consistently. No cecal or colonic ulcerations or storage of carrageenan, or induction of lysosomal enzymes were observed in this model by carrageenan.

Source: FMC Corporation Manchester

(16)

Species: rat **Sex:** male
Strain: Sprague-Dawley
Route of admin.: oral feed
Exposure period: 4 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 50 g/kg diet
Control Group: yes, concurrent no treatment
Method:
Year: 1984 **GLP:** no
Test substance: other TS: iota carrageenan from Sigma Chemical
Remark: Methods: Six male rats were fed a purified diet containing 50 g/kg carrageenan for 4 weeks. Another group received the purified diet untreated. Both groups were sacrificed after the 4 weeks and the cecal content from each animal was removed, weighed and analyzed for azoreductase, nitroreductase, nitrate reductase, beta glucosidase and beta glucuronidase, urease, ammonia content. The total cecal bacterial population was determined also.

Results: Carrageenan significantly increased the weight of both the cecal wall and cecal contents. It also decreased the number of bacteria to 2% of the control value, and decreased cecal ammonia by 76% (probably due to decreased bacterial load). In addition all microbial enzyme activities were decreased in the cecum of the treated vs the control rats. Carrageenan is resistent to attack by the gut microflora.
Source: FMC Corporation Manchester

(17)

Species: rat **Sex:** male
Strain: Sprague-Dawley
Route of admin.: oral feed
Exposure period: 10 days
Frequency of treatment: continuous
Post. obs. period: none
Doses: 5% native carrageenan
Control Group: yes, concurrent no treatment
Method:
Year: 1970 **GLP:** no
Test substance: other TS: from Societe Auby, Neuilly-sur-Seine, France
Remark: Methods: Five rats/sex/group received either a diet containing 5% carrageenan or an untreated diet for 10 days. Food consumption was measured daily, 24 hour fecal samples from each animal were collected on the 10th day and analyzed for 3,6-anhydrogalactose content. Carrageenan content was calculated from this value. Food intakes for the first seven and last three days, and the weight gains for each animal were taken. Animals were sacrificed without necropsy.

Results: Food intakes indicated that there were no meaningful differences between control animals and those

receiving carrageenan. However, the mean weight gain of the control group was significantly higher than that of the test groups. The presence of carrageenan in the diet was readily detected by the increased weight of the feces compared to that of the control group. No toxicological effects were observed in the treated group.

Source: FMC Corporation Manchester

(18)

Species: rat **Sex:** male/female
Strain: Sprague–Dawley
Route of admin.: oral feed
Exposure period: 6 months
Frequency of treatment: continuous
Post. obs. period: none
Doses: 4% carrageenan
Control Group: yes, concurrent no treatment
NOAEL: = 4 %
Method:
Year: 1974 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Two groups consisting of 25 rats/sex each were fed semi–defined diets containing 0 or 4% carrageenan for 6 months. The carrageenan was added to skim milk in the proportion of 1 part to 1 part protein (N X 6.38). The premix was pasteurized, sealed in cans and sterilized at 265 F for 3.68 minutes. The basal diet was modified by replacing skim milk powder and cellulose with the processed milk–carrageenan product. The rats were weighed twice weekly. At intervals 6–8 rats from each of the groups were placed in metabolism cages for 5–7 days for the study of the absorption of nutrients. At autopsy the organs were examined for gross lesions. The lower GI tract was examined carefully by injection of a dye into the tail vein before sacrifice. Mucosal injury would be indicated by the presence of dark blue areas in the mucosa. The cecum and colon were examined microscopically also. At 6 months of study blood was taken and analyzed for prothrombin time and partial thromboplastin time.

Results: Growth and development during the 6 month feeding period was normal for both carrageenan fed and control group rats. During the first month the males gained 6.1 g/day which is in the maximal range for this strain of rats. The utilization of dietary energy for growth (g of gain/100 kcal diet) was also not influenced by the heat processed carrageenan. Cecum and colon tissue were normal, with no evidence of erosions or ulcerations of the mucosal surfaces. There were no statistically significant differences in mean prothrombin time or partial thromboplastin time between carrageenan and cellulose control animals.

Source: FMC Corporation Manchester

(19)

Species: rat **Sex:** male
Strain: Sprague–Dawley
Route of admin.: oral feed
Exposure period: 5 days
Frequency of treatment: continuous
Post. obs. period: 2 days
Doses: 4% in diet alone or mixed with milk and heat processed (same %).
Control Group:
Method:
Year: 1974 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Absorption studies were conducted on unprocessed samples and samples of carrageenan that had been heat sterilized in milk solution. Adult male rats (6–8/group) in metabolism cages were fed a basal diet for 7 days, followed by 5 days of feeding the carrageenan diet, and then 2 more days on the basal diet. Preliminary studies had shown that carrageenan excretion was completed 48 hours after the last feeding. The feces for each 7–day period for each rat were combined, dried, pulverized, and analyzed for carrageenan content.

Results: Both untreated or heat sterilized carrageenan in milk was quantitatively excreted by the rat. The 98% average excretion values were not significantly different from 100% using the "t" test.
Source: FMC Corporation Manchester

(19)

Species: rat **Sex:** male
Strain: Sprague–Dawley
Route of admin.: oral feed
Exposure period: 6 months
Frequency of treatment: continuous
Post. obs. period: none
Doses: 4% carrageenan
Control Group: yes, concurrent no treatment
Method:
Year: 1974 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Three experiments were performed to determine if there was any difference in the rats' protein utilization in the presence of native carrageenan mixed with or without milk products. In one experiment a group of 8 rats received a basal diet containing skim milk powder and carrageenan powder (10% protein, 4% carrageenan), while a control group received the same diet with cellulose powder and the skim milk powder. In a second study the test group received a mixture of carrageenan in canned liquid skim milk commercially sterilized, while the control received only the canned sterilized liquid skim milk with cellulose. The third test was composed of 4 groups of rats; one received

autoclaved liquid skim milk and cellulose (4%) in the diet (control), and the other received the same with carrageenan (4%) added. Autoclaving was excessive when performed for 45 minutes at 15 psi. A third group received skim milk powder with cellulose, unsterilized (control) and the fourth skim milk powder and carrageenan powder, unsterilized. All diets contained 10% milk protein and the carrageenan or cellulose concentration was 4%. Animals were maintained in metabolism cages so that food intake could be recorded, urine and feces collected. Protein efficiency ratios were calculated for animals in all groups.

Results: The protein efficiency ratios indicated that the native carrageenan, carrageenan in skim milk sterilized under manufacturing conditions, or autoclaved carrageenan in skim milk had no effect on the utilization of protein for growth.

Source: FMC Corporation Manchester

(19)

Species: rat **Sex:** male
Strain: Sprague–Dawley
Route of admin.: oral feed
Exposure period: 8 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 4% in diet
Control Group: yes
Method:
Year: 1974 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: The possibility that carrageenan might interfere with the absorption of iron was investigated by determining the effect of dietary carrageenan on the depletion and regeneration of hemoglobin in rats fed a low–iron diet.

Weanling male rats were fed a basal diet, without the ferrous sulfate ingredient, for 1 week. They were then divided into two groups of equal average body weight and hemoglobin level. The experimental group received the processed carrageenan incorporated into the basal diet at the expense of the cellulose. After 4 weeks on the deficient diets the control and carrageenan diets were then fortified with a suboptimal amount of ferrous sulfate (10 mg Fe/kg diet). Hemoglobin values were measured 2 and 4 weeks later.

Results: Anemia was produced after 3 weeks without added iron in both the cellulose and carrageenan groups (6.61, 6.07 g/100 ml). After 2 weeks, iron levels were 8.87 (cellulose) and 8.24 (carrageenan), and after 4 weeks they were 10.89 and 10.65 g/100 ml, respectively. The iron–deficient diet did not increase the severity of the anemia, nor did it impede the regeneration of hemoglobin when iron supplementation was instituted.

Source: FMC Corporation Manchester

(19)

Species: rat **Sex:**
Strain: Sprague-Dawley
Route of admin.: oral feed
Exposure period: 3 months
Frequency of treatment: continuous
Post. obs. period: none
Doses: 4 %
Control Group: yes, concurrent vehicle
NOAEL: = 4 %
Method:
Year: 1974 **GLP:** no
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: The absorption of fat, calcium and total solids was determined in the rats during the third month of a long-term feeding study in which one group received a defined diet containing 20% protein and 4% carrageenan and the other received the same diet with cellulose replacing the carrageenan. Specific methods are not presented.

Results: No differences of statistical significance were found between the groups fed the processed carrageenan or cellulose in percent absorption of total solids, fatty acids or calcium. Therefore, absorption of nutrients from the intestine was not impeded by the presence of the carrageenan.

Source: FMC Corporation Manchester

(19)

Species: rat **Sex:**
Strain: Sprague-Dawley
Route of admin.: oral feed
Exposure period: 6 months
Frequency of treatment: continuous
Post. obs. period: none
Doses: 4% carrageenan
Control Group: yes
NOAEL: = 4 %
Method:
Year: 1974 **GLP:** no
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: Rats on a long-term study were used to evaluate coagulation times after six months of treatment. Control diet contained 4% cellulose and treated diet contained 4% carrageenan. Both prothrombin time and partial thromboplastin times were evaluated.

Results: The mean prothrombin times were 11.5 sec and 11.7 sec for cellulose and carrageenan fed rats, respectively, and similarly the partial thromboplastin times were 27.2 and 26.9 sec for cellulose and carrageenan fed rats.

Source: FMC Corporation Manchester

(19)

Species: rat **Sex:** male/female
Strain: Sprague–Dawley
Route of admin.: oral feed
Exposure period: 1 year
Frequency of treatment: continuous
Post. obs. period: none
Doses: 1 and 5% in diet
Control Group: other: concurrent alphacel
Method:
Year: **GLP:** no data
Test substance: other TS: RE–7063, extract of Hypnea–similar to kappa carrageenan: and RE–7064, Iridaea extract, lies between lambda and kappa carrageenan
Remark: Methods: Fifteen rats/sex were administered either the Hypnea extract or the Iridaea extract in the diet for 1 year at both 1 and 5%. Controls received alphacel in their diets. Body weights were recorded weekly and stools were observed regularly for consistency and checked for presence of occult blood by means of Hematest tablets regularly. Standard clinical hematological and chemical methods were measured and tissues were obtained at necropsy for histology, histochemistry and electron microscopy.

Results: The body weights of both male and female carrageenan fed rats were 20–25% below the 5% alphacel controls after 1 year of study. No other effects were seen which could be attributed to test article administration. No metachromatic material was found in the liver slices of any rats on study. Ultrastructural examination of livers showed no evidence for storage of fibrillar materials. Therefore there was no significant toxicological effect of carrageenan intake on rats fed either 1 or 5% in the diet for 1 year.

Source: FMC Corporation Manchester

(20)

Species: rat **Sex:** male/female
Strain:
Route of admin.: oral feed
Exposure period: 39 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 5% of two different diets
Control Group: yes
Method:
Year: 1985 **GLP:** no data
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: Two strains of weanling rats, Sprague-Dawley (SD) and Osborne-Mendel (OM), were compared in this study. Also two different diets were used: one was Wayne Lab Blox and the other was the standard diet used at the FDA laboratories. Eight groups containing 18 rats each were assigned to the following tests: Group 1 was OM rats fed 5% Alphacel in FDA diet; Group 2 was OM rats fed 5% carrageenan in FDA diet; Group 3 was OM rats fed 5% Alphacel in Wayne diet; Group 4 was OM rats fed 5% carrageenan in Wayne diet; Group 5 was SD rats fed 5% Alphacel in FDA diet; Group 6 was SD rats fed 5% Alphacel in Wayne diet; and Group 8 was SD rats fed 5% carrageenan in Wayne diet. Survival, weekly body weight, clinical signs, food and water consumption and stool consistency were recorded. The presence of fecal occult blood was monitored. Interim sacrifices were done at 30 days (3 rats/sex/group) and at 90 days (5 rats/sex/group), with all rats receiving a complete necropsy. The surviving rats were sacrificed after 38 weeks and examined grossly. Liver was removed, weighed and examined histochemically for presence of carrageenan. It was also paraffin-embedded and examined microscopically.

Results: No deaths occurred. The carrageenan in the Wayne diet caused lower body weights in SD males at 4 weeks and in OM females throughout the experimental period. Feces were soft in rats in all treated groups. Relative liver weights were significantly increased after 39 weeks only in OM rats fed 5% carrageenan in the FDA diet. Both 5% Alphacel and 5% carrageenan diets caused hepatocellular pericentral atrophy. Bile duct proliferation was observed only in livers of rats given carrageenan. These changes were considered to be minor findings especially since there was no retention or storage of carrageenan found in the livers.

Conclusion: No significant toxicity was observed after 39 weeks of administration of carrageenan in two different diets to two different strains of rats.
Source: FMC Corporation Manchester

(13)

Species: rat **Sex:**
Strain:
Route of admin.: gavage
Exposure period: 28 days to 6 months
Frequency of treatment: daily
Post. obs. period:
Doses: 70 mg/kg to 4000 mg/kg/day
Control Group: no data specified
NOAEL: = 4000 mg/kg
Method:
Year: 1970 **GLP:** no
Test substance: other TS: not specified; called 'native' carrageenan
Remark: Methods: Carrageenan was given by gavage to 200 rats for 28 days at dosage levels ranging from 70 to 4000 mg/kg/day. One group of rats was continued on this regimen at 4000 mg/kg/day for 6 months.

Results: The rats grew normally and no evidence of toxicity was noted when they were sacrificed and given a complete necropsy.
Source: FMC Corporation Manchester

(21)

Species: rat **Sex:** male/female
Strain: Sprague–Dawley
Route of admin.: other: either by gavage or in diet
Exposure period: 13 weeks (in feed) and 9 months (by gavage)
Frequency of treatment: continuous
Post. obs. period: none
Doses: 500 mg/kg (by gavage), 5% in diet (in feed)
Control Group: yes, concurrent no treatment
Method:
Year: 1976 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Young rats of both sexes were divided into groups of three and given either distilled water or one of the carrageenans derived from E. spinosum (iota) with molecular weights of 107000 and 145000, at a dosage level of 500 mg/kg/day for 9 months. In a second experiment weanling Sprague–Dawley rats of both sexes were divided into groups and fed control diet or a diet containing 5% of one of three diets derived from C. crispus (kappa or lambda) with molecular weights of 186,000, 200,000 or 214,000 for 13 weeks. At the end of each of these experiments the animals were sacrificed and the livers removed for analysis of carrageenan content. The electrophoretic mobility of carrageenan in the feces was also determined.

Results: No evidence for the presence of carrageenan in liver was found after qualitative examination of samples from rats given iota or kappa/lambda carrageenans of molecular weights of > 107,000. The electrophoretic mobility of carrageenan in the feces was greater than the

electrophoretic mobility of carrageenan fed, indicating that some degradation occurred.
Source: FMC Corporation Manchester

(22)

Species: guinea pig **Sex:** female
Strain: Hartley
Route of admin.: oral feed
Exposure period: 10 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 2%
Control Group: yes, concurrent no treatment
NOAEL: = 2 %
Method:
Year: 1976 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Two groups containing 10 female guinea pigs each were administered the iota fraction of carrageenan with the following intrinsic viscosities of 5.34, 7.51 dl/g. These were mixed into the diet at 2% and administered continuously for 10 weeks. In addition an untreated control group was also run. Weekly body weight and food consumption were measured. At sacrifice the cecum and duodenum were collected, fixed and stained for select enzymes as well as for carrageenan content, and viewed for histopathological changes under light and electron microscopes.

Results: Iota fractions given to guinea pigs in the diet produced no inflammatory response, erosion or ulceration of the cecum. No indication of uptake of carrageenan into the cecal epithelium was observed in any animals on test.
Source: FMC Corporation Manchester

(23)

Species: guinea pig **Sex:** no data
Strain:
Route of admin.: oral feed
Exposure period: 3–5 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 5% in diet
Control Group: no
Method:
Year: 1973 **GLP:** no
Test substance: other TS: carrageenan from Glaxo
Remark: Methods: Three groups containing 7 females, 5 females and 28 males each were administered carrageenan in the diet at a concentration of 5% for 3–5 weeks (first two groups) and for up to 42 days (third group). The third group also received 0.1% neomycin in the diet along with the test material. Two–3 animals in each group were sacrificed sequentially to determine the onset of GI lesions. At sacrifice, the

gastrointestinal tract was removed and examined with a dissecting scope for the presence of lesions. It was then fixed, sectioned and stained for the presence of carrageenan and lysosomal enzymes. Other sections were observed under the electron microscope for histopathologic changes.

Results: Guinea pigs developed multiple pin-point caecal and colonic ulcerations after 3-5 weeks of treatment. Histologically the ulcers consisted of extensive macrophage infiltration of the base over which lay a layer of fibrin. PMN's and lymphocytes were present in appreciable numbers. The epithelium around the ulcer was heavily infiltrated by macrophages, polymorphs and lymphocytes. The addition of neomycin to the diet markedly reduced the population of polymorphonuclear cells in the ulcers but did not affect the apparent incidence of ulcers or the time of their appearance. The data on time of appearance, presence or absence of carrageenan and the lysosomal enzyme levels were not clearly presented in this paper.

Source: FMC Corporation Manchester

(16)

Species: guinea pig **Sex:** female
Strain: Hartley
Route of admin.: oral feed
Exposure period: 7-10 weeks (feed), 2-3 weeks (drinking water)
Frequency of treatment: continuous
Post. obs. period: none
Doses: 2% in feed, 1% in drinking water
Control Group: no
Method:
Year: 1976 **GLP:** no
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: Guinea pigs were divided into groups and fed control diet or a diet containing 2% of one of the iota carrageenans with molecular weights of 107000, and 145000. Feeding continued for 7-10 weeks after which they were killed and samples of liver, feces and bladder urine were taken from each of the 6 animals/group were obtained for analysis.

In two other studies, groups of female guinea pigs were given a carrageenan of the iota type listed above, or a kappa/lambda type derived from C. crispus with a molecular weight of 275000, in the drinking water for 2-3 weeks at a concentration of 1%. Samples of liver and feces were examined at termination.

Results: Carrageenan was found in the livers of guinea pigs receiving carrageenans of 107000 and 145000 molecular weights in the drinking water. No carrageenan was found in the livers of guinea pigs that received the 275000 MW kappa/lambda type in drinking water. Feces contained the bulk of the carrageenan fed, but the molecular weights of the carrageenan in the feces were 100,000 or less,

indicating that some metabolism had taken place. No carrageenan was detected in the urine.

Source: FMC Corporation Manchester

(24)

Species: guinea pig **Sex:** male
Strain:
Route of admin.: drinking water
Exposure period: 20-30 days
Frequency of treatment: continuous
Post. obs. period: none
Doses: 1% carrageenan
Control Group: yes, concurrent no treatment
Method:
Year: 1969 **GLP:** no
Test substance: other TS: J. W. Cumming & Son Ltd, Salford, UK
Remark: Methods: Groups of 10 male guinea pigs each received either 1% carrageenan or untreated water (control). The animals were killed between 20 and 30 days after the start of the experiment and the Gastrointestinal tract was entirely removed, fixed and examined histologically.

Results: Two of the four animals receiving carrageenan that were killed after 20 days showed multiple ulcerative lesions in the caecum. In the remaining six animals traces of occult blood were present from day 23 on. These animals had ulcerative lesions in the caecum and two had lesions extending into the colon for a short distance. No lesions were seen in the control.

Source: FMC Corporation Manchester

(25)

Species: guinea pig **Sex:** female
Strain: Hartley
Route of admin.: drinking water
Exposure period: 2 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 1%
Control Group: yes, concurrent no treatment
Method:
Year: 1976 **GLP:** no
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: Four groups of female guinea pigs containing 5 pigs each were administered drinking water containing 1% carrageenan fractions kappa, lambda or iota with the following viscosities: kappa - 11.95 dl/g; lambda - 10.250 dl/g; iota - 5.34, 7.51 dl/g. One group received distilled water and was used as the control. These mixtures were admininstered continuously for 2 weeks and the animals were then sacrificed, their duodenum and cecum observed grossly before removal for histological examination. These organs were stained for lysosomal enzymes and for

carrageenan content and were examined under both light and electron microscope. Body weights and water consumption were measured in-life also.

Results: There were no effects observed in any group of pigs receiving any of the kappa or lambda fractions of carrageenan. Also no effects were observed in the groups receiving the iota fractions with 7.51 dl/g intrinsic viscosity. The other iota sample was absorbed and retained in cecal lamina proprial and submucosal macrophages. Histopathological changes in the cecum were epithelial thickening, slight erosion, cellular infiltration and crypt abscesses, and occasional ulceration of the cecal mucosa. No changes were found in the duodenum of any animal examined. This work supports the suggestion that the ulcerogenic process in the guinea pig cecum is initiated by the uptake of carrageenan into lysosomes of lamina proprial macrophages which stimulates the release of lysosomal enzymes, subsequently damaging the surrounding tissue. Fractions not absorbed will therefore not cause cecal epithelial damage.

Source: FMC Corporation Manchester

(23)

Species: guinea pig **Sex:** male
Strain:
Route of admin.: drinking water
Exposure period: 5 months
Frequency of treatment: continuous
Post. obs. period: none
Doses: 1% solution in drinking water; < 0.15 g/100g BW
Control Group: yes, concurrent no treatment
Method:
Year: 1970 **GLP:** no
Test substance: other TS: derived from Eucheuma spinosum
Remark: Methods: Fifteen male albino guinea-pigs were administered a 1% aqueous solution of carrageenan freshly prepared each day and supplied ad libitum as drinking water over a period of 5 months. The daily consumption of carrageenin per animals was less than 0.15 g/100g body weight. Body weights and food consumption were measured and at sacrifice the large bowel was emptied of feces and carefully examined using transmitted light. Control animals (no information on number) ran parallel to the treated for comparison purposes.

Results: The average monthly gain in body weight among the carrageenan fed animals was 125 g compared with 135 among the controls (no statistics performed). In all animals fed carrageenan, multiple focal ulcers were present in the cecum and in four the lesions had extended distally beyond the cecum for about 3 cm. Microscopically the ulcers involved mainly the mucosa and showed subacute or chronic inflammatory reaction. Controls showed no similar lesions.

Source: FMC Corporation Manchester

(26)

Species: monkey **Sex:** male/female
Strain: other: Macaca mulatta
Route of admin.: drinking water
Exposure period: 7–11 weeks
Frequency of treatment: continuous
Post. obs. period: up to 11 weeks
Doses: approximately 1.3 g/kg/day
Control Group: yes, concurrent no treatment
NOAEL: = 1300 mg/kg
Method:
Year: 1973 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Three animals/sex received the test material in drinking–water as a 1% solution. Two animals were sacrificed after 7 and 11 weeks, respectively, and the remaining four were put back on plain tap–water and allowed to recover for 11 weeks. These four were then used for a dose–range finding study, using escalating daily doses of carrageenan ranging from 50 to 1250 mg/kg daily for up to 12 weeks. Dosing was by gavage during this phase of the study. The highest dose was given for 8 weeks. After this second period of exposure all four monkeys were killed. Weight gain, fecal occult blood, clinical signs, were recorded regularly, and hematological parameters were measured prior to termination. At sacrifice the gastro–intestinal system was removed and examined thoroughly. Sections of the cecum, ileocolic valve, colon and adjacent lymph nodes were sectioned and examined microscopically.

Results: The substantial quantities of carrageenan (kappa) ingested did not appear to affect the animals adversely. They continued to gain weight and remained in good condition. Of the 2 animals sacrificed at 7 and 11 weeks the female had a grossly normal gastro–intestinal tract. Microscopic examination of all portions of the GI tract revealed no abnormality. The other four monkeys that were used for the dose–escalation study remained normal throughout the dosing and the gross and microscopic examination of the intestinal tract revealed no change attributable to the administration of carrageenan.

Conclusion: No significant treatment related effects were observed in monkeys receiving 1.3 g/kg/day of carrageenan for up to 11 weeks.
Source: FMC Corporation Manchester

(27)

Species: monkey **Sex:** male/female
Strain: other: Macaca mulatta
Route of admin.: drinking water
Exposure period: 7–11 weeks
Frequency of treatment: continuous
Post. obs. period: 11 weeks
Doses: 1% in drinking water, 1.3 g/kg/day dosage
Control Group: yes, concurrent no treatment
NOAEL: = 1.3 mg/kg
Method:
Year: 1972 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Three monkeys/sex were administered kappa carrageenan in the drinking water at a concentration which produced a dosage of 1.3 g/kg/day for up to 11 weeks. Two monkeys/sex were used as controls. One carrageenan dosed female was sacrificed after 7 weeks of dosing, and one carrageenan dosed male after 11 weeks of dosing. The other four monkeys were placed on plain water and held for a recovery period of 11 weeks before sacrifice. Prior to death a solution of horseradish peroxidase was injected into the tail vein. At autopsy the liver was removed and sections fixed, while frozen sections were prepared of other parts. Stains to detect lysosomal enzymes, peroxidases and beta glucuronidase were used. Small blocks of liver tissue were examined with electron microscopy.

Results: The livers of monkeys given carrageenan were indistinguishable from those of the control group. The distribution, number, size and enzyme activity of the lysosomes in the hepatocytes was normal. The same was true of both the parenchymal and Kupffer cells, where no influence of carrageenan was apparent. Therefore, carrageenan did not give rise to stored material in reticuloendothelial cells or in hepatocytes.
Source: FMC Corporation Manchester

(28)

Species: monkey **Sex:** male/female
Strain: other: Macaca mulatta
Route of admin.: gavage
Exposure period: 7.5 years
Frequency of treatment: 6 days/week
Post. obs. period: none
Doses: 0, 50, 200, 500 mg/kg/day
Control Group: yes
NOAEL: = 500 mg/kg
Method:
Year: 1983 **GLP:** no data
Test substance: other TS: source not stated
Remark: Methods: Four groups containing 5 monkeys/sex each were administered carrageenan at levels of 0, 50, 200, or 500 mg/kg for 6 days/week for 7.5 years. Carrageenan was administered via stomach tube or hand formed diet pellets. Parameters measured were body weight, food consumption, clinical signs and stool consistency, which were measured weekly and hematology, clinical chemistry and fecal occult blood were performed at selected intervals. Liver biopsies were obtained from high dose animals once/year. At study termination, selected organs of all survivors were weighed after gross postmortem examinations and histopathological examination were conducted on all animals. Liver, intestines, cecum and lymph nodes from each animal were examined by both light and electron microscopy for pathological lesions and the presence of carrageenan.

Results: Long term ingestion of carrageenan had no adverse effects on these monkeys. Dose related trends toward fecal softening and increased but variable fecal occult blood were observed during the study. Carrageenan was not present in the liver, lymph nodes, cecum or intestine following 7.5 years of exposure.
Source: FMC Corporation Manchester

(29)

Species: monkey **Sex:** male/female
Strain: other: Macaca mulatta
Route of admin.: gavage
Exposure period: 9 months
Frequency of treatment: daily
Post. obs. period: none
Doses: 50, 200, 500 mg/kg
Control Group: no
Method:
Year: 1976 **GLP:** no
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: Rhesus monkeys of both sexes were given carrageenan daily in doses of 50, 200, or 500 mg/kg by stomach tube. Overnight collections of feces were obtained after 11 months. Overnight collections of urine were obtained from two of the monkeys on the high dose after 15 months. Fecal samples were examined qualitatively by gel electrophoresis, and mobilitites of carrageenans found were reported as compared to mobilities of the original substance.

Results: The carrageenans contained in the feces had molecular weights of 100,000 or less. Since the original carrageenan administered had a molecular weight of 185,000, it appears that some metabolism had taken place in the gut. Mobility of those found in the feces (0-0.67) was greater than the mobility of the standard sample (0-0.09), indicating that the feces contained some carrageenan of lower molecular weight than the material administered.
Source: FMC Corporation Manchester

(22)

Species: other: baboon **Sex:** male/female
Strain:
Route of admin.: other: in formulas fed
Exposure period: 112 days
Frequency of treatment: 5 times/day for first 14 days, 4 times/day for next 14 days, 3 times/day for the next 56 days and 2 times/day for the next 28 days
Post. obs. period: none
Doses: no added carrageenan, 255 mg/liter added, 1220 mg/liter added
Control Group: yes, concurrent no treatment
Method:
Year: 1977 **GLP:** no
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: Three groups of newborn baboons containing 3 male and 5 females each were taken from their mothers between 18 and 24 hours after delivery and were administered a basic defined infant formula, containing either no (control), 255 mg/liter or 1220 mg/liter carrageenan for 112 days. Baboons in all groups were fed on the same regimens depending upon age. Body weight was measured daily and organ weights were taken at autopsy. Hematological endpoints were measured at

birth, monthly, and before autopsy by heel stick. Blood chemistries were evaluated just before autopsy. At termination the alimentary tract was removed from each animal and examined under dissecting microscope. Fourteen blocks for histological sections were taken from standard sites in the alimentary tract, including seven from the colon. All major viscera as well as central nervous system were fixed and examined microscopically.

Results: No biologically significant difference was found for total formula consumed by each animal from birth through day 105, between control and treated groups. Mean growth was similar across groups also. No changes were observed in hematology, clinical chemistry, or the macroscopic or microscopic appearance of the gastrointestinal tract or any other organ.

Source: FMC Corporation Manchester

(30) (31)

Species: pig **Sex:** male/female
Strain: other: Danish Landrace
Route of admin.: oral feed
Exposure period: 83 days
Frequency of treatment: daily
Post. obs. period: none
Doses: 50, 200 and 500 mg/kg/day as jelly mixed with diet, or 500 mg/kg/day carrageenan and 1 g/kg/day mixed with powdered diet
Control Group: yes, concurrent vehicle
NOAEL: = 500 mg/kg
Method:
Year: 1973 **GLP:** no
Test substance: other TS: Copenhagen Pectin Factory, Denmark
Remark: Methods: Groups of three pigs/sex were given carrageenan for 83 days according to body weight. Three groups were given carrageenan as a jelly mixed with the diet pellets at levels of 50, 200 or 500 mg/kg/day. A fourth group received carrageenan (500 mg/kg/day) and sucrose (1 g/kg/day) mixed with the powdered diet. A control group of three pigs/sex was given the corresponding amount of sucrose. In the data for clinical chemistry, control values were included from an extra four pigs of similar age and maintained under identical conditions. These pigs were not used for any other purposes in this study. Body weight was recorded weekly and food intake was recorded twice daily. Blood samples were collected 16 and 9 days prior to and 5, 19, 47 and 75 days after the commencement of carrageenan administration. Hematology and some clinical chemistry parameters were examined. Urine samples were collected during the last 14 days of treatment and analyzed. Pigs were sacrificed, gross abnormalities were noted and 13 organs were weighed. Samples from all primary organ systems were examined microscopically. Bacteriological examination was performed on the flora of the jejunum, cecum, colon and ampulla of the rectum from all animals of the vehicle control group, and the group given 500 mg carrageenan/kg/day

as a jelly.

Results: The appearance and behaviour of the pigs remained normal throughout the experiment. There were no significant findings in any of the parameters examined that could be test material related. No ulcerations or erosions were seen in the gastro-intestinal tract.

The total count of aerobic bacteria/g intestinal contents was significantly reduced in the colon and rectum of the pigs given carrageenan compared with the control values. The number of Lactobacilli in the rectum was reduced in the group given 500 mg/kg. In three pigs (one receiving 200 and 2 receiving 500 mg/kg/day) thick and uneven areas were seen in the colonic mucosa near the cecum. Histologically these areas showed infolding of intact epithelium. The lamina propria of the mucosa was thickened with diffuse infiltration of macrophages and lymphocytes. These slight changes were not considered to be a signficant toxic effect. Generally a shift was seen in the cellular infiltration pattern of the mucosa of the large intestine of pigs from all groups given carrageenan.

Source: FMC Corporation Manchester

(32)

Species: rodent **Sex:** male
Strain:
Route of admin.: oral feed
Exposure period: 30 days
Frequency of treatment: continuous
Post. obs. period: none
Doses: 5, 20 or 50 g/kg diet
Control Group: yes, concurrent no treatment
LOAEL: = .5 %
Method:
Year: 1985 **GLP:** no
Test substance: other TS: iota carrageenan mostly; some kappa. All food grade.
Remark: Methods: Male Sprague-Dawley rats, MF1 mice and DSN hamsters were fed a fiber-free purified diet or that diet supplemented with 5, 20 or 50 g/kg carrageenan for 30 days. Bile samples were obtained from rats by bile duct cannulation and analyzed for immunoglobulin A (IgA) antibody. All animals were killed and the entire contents of the cecum was weighed, analyzed for microbial biotransformation activities under anaerobic conditions. The total bacterial numbers were determined, as was recovery of viable organisms. The concentration of carrageenan in the rat hindgut was obtained also.

Results: Carrageenan treatment produced caecal enlargement in all the rodent species, yet significantly decreased the concentration of bacteria per gram of cecal content. Enzyme activities per gram of cecal content were significantly decreased in the rat. Less consistent results were observed

in the hamster and mouse. Carrageenan significantly decreased the concentration of enterobacteria, staphylococci, streptococci, lactobacilli, facultative anaerobes and total microscopic count in the rat cecum but did not exert any effect on bacterial viability in vitro. When present at 50 g/kg diet carrageenan increased the agglutination response of the IgA specific for the hindgut microflora, but had no effect upon biliary IgA antibody concentrations.

Carrageenan treatment had no effect on final body weight in the rat, mouse or hamster.

Source: FMC Corporation Manchester

(33)

5.5 Genetic Toxicity 'in Vitro'

Type: Ames test
System of testing: Salmonella typhimurium stratin TA 100
Concentration: 25, 50, 100 mg/ml natural carrageenan, 25 mg/ml refined carrageenan
Metabolic activation: without
Result: negative
Method: other: No information on procedure included. Reference is made to Ames, 1971 publication.
Year: 1993 **GLP:** no data
Test substance: other TS: carageenan, PNG and refined
Remark: Methods: The Ames test without S–9 mix was employed to assess the mutagenicity potential before metabolic activation.

Results: No mutagenicity was observed in this assay.

Source: FMC Corporation Manchester

(34)

Type: Ames test
System of testing: TA 100, TA 98 Salmonella typhimurium
Concentration: 8.9 and 26.7 mg/plate
Metabolic activation:
Result: negative
Method: other: Tester strains were preincubated with S9 mix with or without 1uM of norharman, a comutagen. Simultaneous cytotoxicity tests showed no cell killing effects of test compounds at the doses examined.
Year: 1984 **GLP:** no data
Test substance: as prescribed by 1.1 – 1.4
Source: FMC Corporation Manchester

(35)

Type: Ames test
System of testing: Salmonella typhimurium
Concentration: 0.55%, 1.10%, 2.20%
Metabolic activation: with and without
Result: negative
Method: other: solvent was DMSO; activation preparations from mice, rats and monkeys were tested. Bacterial strains tested were TA-1535, TA-1537, TA-1538.
Year: 1975 **GLP:** no
Test substance: other TS: calcium carrageenan
Remark: Results: The test material suspended in DMSO without activation did not exhibit genetic activity in this assay. It also was non genotoxic when the metabolic activation systems derived from mouse, rat or monkey were added to the plates. Therefore, in this assay there is no indication that the test material posesses mutagenic potential.
Source: FMC Corporation Manchester

(36)

Type: Ames test
System of testing: S. typhimurium strains TA 1535, TA 1537, TA 1538; S. cerevisiae strain D4
Concentration: bacteria and yeast: 0.5%, 1.0% and 2.0%
Metabolic activation: with and without
Result: negative
Method:
Year: 1975 **GLP:** no
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: The test material was dissolved in saline and then tested in both plate tests (nonactivation) and suspension tests (both with and without activation) in this reverse mutation assay in both bacteria and yeast. Activation systems were prepared from the ICR mouse, Sprague-Dawley rat, and Macaca monkey and each was tested separately. Appropriate positive and negative controls were used in all cases.

Results: The test compound did not exhibit genetic activity in any of the in vitro assays employed in this evaluation.
Source: FMC Corporation Manchester

(37)

Type: Mammalian cell gene mutation assay
System of testing: human embryonic lung cultures (WI–38)
Concentration: 8, 80, 800 mcg/ml in saline
Metabolic activation: without
Result: negative
Method:
Year: 1972 **GLP:** no
Test substance: other TS: sodium carrageenan
Remark: Methods: Tissue cell cultures of human embryonic lung tissue were prepared and the test compound was added at three different dose levels using three bottles for each level. Cells were incubated at 37 C and examined twice daily to determine when an adequate number of mitoses were present. They were then harvested (usually 24–48 hours after planting), centrifuged and fixed, then prepared on slides for microscopic examination. Positive (TEM) and negative controls were run concurrently. Slides were examined for chromosomal damage.

Results: No significant aberrations in the anaphase chromosomes of human tissue culture cells were observed when tested at the dosage levels employed in this study.
Source: FMC Corporation Manchester

(37)

Type: Yeast gene mutation assay
System of testing: Saccharomyces cerevisiae, strain D4
Concentration: 0.2438%, 0.4875%, and 0.9750% in DMSO
Metabolic activation: with and without
Result: negative
Method: other: Range–finding cytoxicity studies were used to determine the final concentrations tested. Tests were run with and without activation systems extracted from mouse, rat and monkey.
Year: 1975 **GLP:** no
Test substance: other TS: Calcium carrageenan
Remark: Results: The compound did not exhibit genetic activity in this in vitro yeast assay.
Source: FMC Corporation Manchester

(36)

Type: other: bacterial gene mutation assay and yeast gene mutation assay
System of testing: TA 1530 and G–46 S. typhimurium; D–3 Saccharomyces
Concentration:
Metabolic activation: without
Result: negative
Method:
Year: 1972 **GLP:** no
Test substance: other TS: sodium carrageenan
Remark: Methods: Cultures of S. typhimurium histidine auxotrophs G–46 and TA 1530 were plated on media. The test compound was then added to the plate, either in the form of a microdrop of solution applied to a small filter paper disc resting on the agar or a small crystal applied directly to the agar. Tenfold serial dilutions of the culture were employed and plated so as not to miss the optimum cell density for mutant growth. Mutant colonies were observed and scored.

Strain D–3 Saccharomyces cells at proper dilutions were shaken with the test compound, diluted, and then plated at 59% survival level or above. Red sectors were then scored and frequency calculated after suitable incubation. Negative and positive (EMS) controls were run concurrently.

Results: The test material was not mutagenic in this assay.
Source: FMC Corporation Manchester

(37)

5.6 Genetic Toxicity 'in Vivo'

Type: Cytogenetic assay
Species: rat **Sex:** male
Strain: Sprague–Dawley
Route of admin.: gavage
Exposure period: single dose
Doses: 30, 2500, 5000 mg/kg
Result:
Method:
Year: 1972 **GLP:** no
Test substance: other TS: Sodium carrageenan
Remark: Methods: Nine groups containing 5 male rats each were administered the test material at dosage levels of 50, 2500 and 5000 mg/kg. Another three groups of 3 rats each were dosed with saline alone and were considered the negative controls. One positive control group containing 5 rats received TEM. Colcemid was administered and rats were killed at 6, 24, and 48 hours after dosing (positive control, 48 hours only) and the bone marrow was prepared for microscopic examination of the chromatin.

Results: The compound produced no detectable significant aberration of the bone marrow metaphase chromosomes of rats when administered orally at the dosage levels employed in

this study.
Source: FMC Corporation Manchester

(38)

Type: Cytogenetic assay
Species: rat **Sex:** male
Strain: Sprague–Dawley
Route of admin.: gavage
Exposure period: 5 days
Doses: 30, 2500, 5000 mg/kg/day
Result:
Method:
Year: 1972 **GLP:** no
Test substance: other TS: sodium carrageenan
Remark: Methods: Three groups containing 5 rats each were administered the test material by gavage at dosage levels of 30, 2500 and 5000 mg/kg/day for 5 days. Four hours after the last dosing and two hours prior to killing each animal was given 4 mg/kg colcemid i.p. to arrest bone marrow cells in C–mitosis. The femur was removed and bone marrow cells prepared for microscopic examination. Three rats were administered saline and served as the controls.

Results: The compound produced no detectable significant aberration of the bone marrow metaphase chromosomes of rats when administered orally at dosage levels employed here.
Source: FMC Corporation Manchester

(37)

Type: Dominant lethal assay
Species: rat **Sex:** male/female
Strain: Sprague–Dawley
Route of admin.: gavage
Exposure period: single dose
Doses: 30, 2500, 5000 mg/kg
Result:
Method:
Year: 1972 **GLP:** no
Test substance: other TS: sodium carrageenan
Remark: Methods: Ten male rats were assigned to each of 5 groups; 3 dose levels of 30, 2500, 5000 mg/kg carrageenan, a positive control (TEM) and negative solvent control. Following treatment the males were sequentially mated (5 days each) to 2 females per week for 8 weeks. Each pair remained with a male for 5 days. Fourteen days after separating from the male, females were killed and the uterus was examined for deciduomata, late fetal deaths and total implantations. Ovaries were examined and corpora lutea counted.

Results: The test material is considered to be non–mutagenic in this assay with the dosages employed.
Source: FMC Corporation Manchester

(37)

Type: Dominant lethal assay
Species: rat **Sex:**
Strain: Sprague–Dawley
Route of admin.: gavage
Exposure period: 5 daily doses
Doses: 30, 2500, 5000 mg/kg/day
Result:
Method:
Year: 1972 **GLP:** no
Test substance: other TS: sodium carrageenan
Remark: Methods: Ten male rats were assigned to each of 5 groups including three dose levels of carrageenan, a positive and a negative control. Treated groups received the test material orally by gavage at dosage levels of 30, 2500, and 5000 mg/kg/day for 5 consecutive days. The TEM (positive control) material was administered intraperitoneally. Following treatment the males were sequentially mated to 2 untreated females per week for 7 weeks. Each pair of females was housed with a male for 5 days and then 14 days after separating from the male they were killed. The uterus was examined for deciduomata late fetal deaths and total implantations. Ovaries were examined for corpora lutea.

Results: The test material was not mutagenic in this assay.
Source: FMC Corporation Manchester

(37)

Type: Micronucleus assay
Species: mouse **Sex:**
Strain: Swiss Webster
Route of admin.: gavage
Exposure period: two doses 24 hours apart
Doses: 625, 1250, 2500 mg/kg
Result:
Method: other: The test substances were administered twice by oral gavage 24 hours apart. Six hours later the mice were killed , femurs removed and bone marrow cells flushed out using fetal calf serum. Cells were collected and mounted on slides
Year: 1993 **GLP:** no data
Test substance: other TS: carageenan PNG and refined was obtained from FDA
Remark: Results: Carageenan did not alter the chromatin material of the bone marrow cells. No micronucleated polychromatic erythrocytes were found. Carageenan did not produce a mutagenic response in this assay.
Source: FMC Corporation Manchester

(34)

Type: Micronucleus assay
Species: mouse **Sex:**
Strain: Swiss Webster
Route of admin.: gavage
Exposure period: 2 doses 24 hours apart
Doses: 625, 1250 and 2500 mg/kg twice 2 hours apart
Result:
Method:
Year: 1993 **GLP:** no data
Test substance: other TS: Carageenan PNG and refined obtained form FDA
Remark: Methods: Along with the carageenan one of three genotoxins were also administered. These included dimethylhydrazine, dimethylnitrosamine and benzo(a)pyrene. Bone marrow cells were then obtained and read for micronuclei.

Results: The incidence of micronuclei, and thus the chromosome breaking effects, of these genotoxins were significantly reduced by administration of the carageenan. Because these genotoxins work only after they are metabolized by the liver, these results indicate that the carageenan in some way interferes with their metabolism or in some way traps the alkylating species.
Source: FMC Corporation Manchester

(39)

Type: other: Host Mediated Assay
Species: mouse **Sex:** no data
Strain: Swiss Webster
Route of admin.: gavage
Exposure period: no data
Doses: 625, 1250, 2500 PNG, 700 refined
Result:
Method: other: specifics not available. Carrageenan was administered by gavage. The indicator organism, S. typhimurium G46 was injected intraperitoneally and later observed for mutation frequency. s
Year: 1993 **GLP:** no data
Test substance: other TS: Carageenin PNG and refined obtained from FDA
Remark: Methods: Both positive (benzo (a) pyrene) and negative controls were also run.

Results: The mutation frequency of the indicator organism which was injected into the peritoneal cavity was not affected by carageenan samples which were given by oral gavage. This indicates that metabolites of carageenan did not interact with DNA of the indicator organism.
Source: FMC Corporation Manchester

(39)

Type: other: Host-mediated assay
Species: mouse **Sex:** male
Strain: ICR
Route of admin.: gavage
Exposure period: single dose
Doses: 30, 2500, 5000 mg/kg
Result:
Method:
Year: 1972 **GLP:** no
Test substance: other TS: sodium carrageenan
Remark: Methods: Ten mice/group were administered the test material by gavage once at dosage levels of 30, 2500, and 5000 mg/kg. Solvent (saline) and positive control groups were also included. All animals received a single i.p. dose of one of two indicator organisms, either Salmonella (TA 1530) or Saccharomyces (D-46) immediately after dosing. three hours later, each animal was killed and 2 ml sterile saline was introduced intraperitoneally. As much fluid as possible was then removed from the peritoneal cavity and cultured, and the mutation frequiency (bacteria) or recombinant frequency (yeast) for each was calculated. The induction of reverse mutation was determined with the Salmonella, and mitotic recombination was determined with the yeast.

Results: This compound was non-mutagenic at the dosage levels used in this study.
Source: FMC Corporation Manchester

(37)

Type: other: Host-mediated assay
Species: mouse **Sex:** male
Strain: ICR
Route of admin.: gavage
Exposure period: 5 consecutive daily doses
Doses: 30, 2500, 5000 mg/kg/day
Result:
Method:
Year: 1972 **GLP:** no
Test substance: other TS: sodium carrageenan
Remark: Methods: Ten mice/group were administered the test material at dosage levels of 30, 2500, 5000 mg/kg/day for 5 consecutive days. Similar groups received saline (by gavage), or EMS i.m. as postive control in the yeast assays. Immediately after dosing the animals received 2 ml of either Salmonella (TA 1530) or Saccharomyces (D-46) i.p., and three hours later each animal was killed and 2 ml sterile saline was introduced i.p. As much fluid as possible was then removed from the peritoneal cavity, plated and the mutation frequency (for bacteria) or recombinant frequency (for yeast) was calculated for each sample.

Results: This compound was non-mutagenic at the dose levels used in this study.
Source: FMC Corporation Manchester

(37)

5.7 Carcinogenicity

Species: rat **Sex:** male
Strain: Fischer 344
Route of admin.: oral feed
Exposure period: 3 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: about 0.8 g/day in groups 1 and 3
Result:
Control Group: yes
Method:
Year: 1988 **GLP:** no data
Test substance: other TS: source not stipulated
Remark: Methods: Two groups consisting of six male F344 rats each were administered weekly injections (subcutaneous) of dimethylhydrazine (DMH) at a dosage of 20 mg/kg for 3 consecutive weeks. At the same time one of these groups received carrageenan in the diet which resulted in the intake of 0.83 mg/kg/day, and the other group received untreated diet. At the same time, two other groups of rats did not receive the DMH injections but one was on diet containing carrageenan and the other group received only untreated diet and acted as the negative control. Daily food consumption and fecal output were measured for the last 4 days of the experiment. Feces were analyzed for carrageenan content and bile acid content.

Results: No difference was found in body weight and food intake among the groups. The rats fed the carrageenan diet excreted more feces than those fed control diet. In both groups receiving carrageenan 97-98% of the feces was comprised of intact carrageenan. Significant increased concentration of lithocholic acid (LCA) and decreased concentration of total bile acids as well as deoxycholic acid (DCA) and hyodeoxycholic acid-like substance were found in the group receiving carrageenan and DMH as compared to those given DMH alone. LCA and DCA are secondary bile acids that have been shown to have a strong tumor-promoting activity. Thus these findings show that the promoting effect of carrageenan on colon tumorigenesis by DMH is probably mediated by increased excretion of the secondary bile acid LCA. Therefore carrageenan itself is probably not the promotor but in the diet it alters the physicochemical environment in the rat intestine resulting in changes in bile acid secretions which act as the promotors for colon cancer induced by DMH.
Source: FMC Corporation Manchester

(40)

Species: rat **Sex:** male
Strain: Fischer 344
Route of admin.: oral feed
Exposure period: 24 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: diet contained 6% carrageenan, replacing cornstarch: intake was approximately 0.8 g/day.
Result:
Control Group: yes
Method:
Year: 1986 **GLP:** no data
Test substance: other TS: not stipulated
Remark: Methods: Four groups containing either 20 (Gr. 1 & 2) or 15(Gr. 3 & 4) rats each were used in this study. Group 1 and
2 received weekly sc injections of DMH at 20 mg/kg for 16 weeks. Group 1 was fed diet containing carrageenan and Group 2 received control diet. Groups 3 and 4 received no DMH, but Group 3 received the diet containing carrageenan and Group 4 received the control diet. Body weight and food intake was estimated weekly. Feces from rats were dried and the weights for 3 days were estimated. Colonic mucosa, liver and blood plasma were collected and along with feces were assayed for beta-glucuronidase activity.

Results: Fecal volume was higher in the groups receiving carrageenan diets than in the controls. The number of tumors per rat was signficantly higher in Gr. 1 (DMH and carrageenan diet) than in Gr. 2 (DMH and control diet). Therefore fecal bulk did not show a protective effect against the development of colonic tumors. The location of the tumors was different, with Gr. 1 producing them in the proximal colon and Gr. 2 in the distal colon. No tumors were found in Groups 3 or 4 not receiving DMH. There were no signficant differences in the beta-glucuronidase activities in the samples of liver, plasma and colonic mucosa examined. However, this enzyme activity was significantly lower in those groups receiving the carrageenan (Gr. 1 and 3) than in those on the control diet. Therefore, the beta-glucuronidase activity did not seem to be related to the tumor-enhancing effect of carrageenan.
Source: FMC Corporation Manchester

(41)

Species: rat **Sex:** male/female
Strain: other: MRC
Route of admin.: oral feed
Exposure period: lifetime
Frequency of treatment: continuous
Post. obs. period: none
Doses: 5%, 2.5%, 0.5%
Result:
Control Group: yes, concurrent no treatment
Method:
Year: 1980 **GLP:** no data
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: MRC outbred rats were divided into three test groups and given food containing carrageenan (kappa) ad libitum daily for life. Groups of 30/sex were administered diet containing 5%, 2.5% and 0.5% and an untreated control group containing 100/sex were maintained on normal pelleted diet. Food consumption was measured biweekly for a 2–day period. Animals were allowed to die spontaneously or were killed when moribund. Complete autopsies were performed on all animals after death. Histological specimens were taken from all visceral organs and from all tumors and gross lesions. All segments of the GI tract were examined carefully for lesions.

Results: Average daily carrageenan intake was 4022 mg/kg (5 %), 1998 mg/kg (2.5%), 360 mg/kg (0.5%). No clinical signs of excessive toxicity, such as significant body weight reduction were observed. Animals occasionally developed a soft stool or diarrhea. The number of tumor–bearing animals, tumors per tumor–bearing animal and the overall incidence and average latency periods did not differ significantly among test and control groups. From these results carrageenan did not demonstrate a carcinogenic effect in either sex.
Source: FMC Corporation Manchester

(42)

Species: rat **Sex:** male/female
Strain:
Route of admin.: oral feed
Exposure period: 24 months
Frequency of treatment: continuous
Post. obs. period: none
Doses: 1, 5, 15, 25% in feed
Result:
Control Group: yes, concurrent no treatment
Method:
Year: 1959 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Five groups containing 5 rats/sex each were administered carrageenan in the diet at levels of 1, 5, 15, and 25% for up to 24 months. Body weight, food and water consumption were measured during the experimental period, and at death all rats received complete necropsy. Organs were examined microscopically.

Results: Animals consuming diets containing the different levels of carrageenan lived as long as those eating the control diet. Mean body weight was reduced in the highest dosage group only and this was considered to be due to decreased intake of essential nutrients and not from the carrageenan directly.

Mean weekly intake of carrageenan during the experiment were 1, 5, 18 and 30 g/week in the 1%, 5%, 15%, and 25% dietary groups respectively. The mean weekly water intake was 28, 8, 5, and 4 g/g carrageenin, respectively. This decrease with increasing dosage was probably due to the physical limit in the volume of food that could be handled by the gastro–intestinal tract.

The rats consuming the diet containing the 25% levels of carrageenin had lesions suggesting hepatic cirrhosis which could indicate some dietary deficiency or insufficiency. No single lesion was consistently found in the rats of all groups.

Carrageenan had no significant adverse effects in rats when fed in the diet at high levels for 24 months, other than effects due to nutritional deficiency from the excessively high dietary concentrations (>5%).
Source: FMC Corporation Manchester

(43)

Species: rat **Sex:** female
Strain: Fischer 344
Route of admin.: oral feed
Exposure period: 40 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 15% of diet
Result:
Control Group: yes, concurrent no treatment
Method:
Year: 1978 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Weanling rats (5 weeks of age) were divided into 4 groups, two containing 30 rats and two containing 15 rats. A semipurified diet was fed to all rats. In two groups, one containing 30 rats another containing 15 rats, carrageenan was added to the diet at 15%. When the rats were 7 weeks old the rats in the larger groups received 1 weekly s.c. injection of azoxymethane (AOM) for 10 weeks (8 mg/kg/week) while continuing to receive either the carrageenan or control diet. Body weight was measured throughout the in–life phase. After 40 weeks, all rats were sacrificed and necropsied. All organs, including the intestines, were examined grossly and histologically for the number and types of tumors.

Results: The animals fed a 15% carrageenan diet showed a 10–20% lower body weight compared with those fed a control diet. No tumors were found in the colon or in other organs of rats fed the control (no carrageenan) diet. One rat receiving the carrageenan diet but not the AOM showed one colon tumor. The incidence of colon tumors (total tumors per tumor–bearing rat) was higher in AOM–treated animals fed carrageenan diet compared with the incidence of colon tumors in those fed a control diet. All rats on the carrageenan diet had more multiple colon tumors than did those on a control diet.

Conclusion: The rats fed a diet containing 15% carrageenan were more susceptible to colon tumor induction by AOM than were rats fed a diet without carrageenan.

The significance of this finding to man is questionable. The dosage level of the carrageenan administered in this study (15% of diet) is very high. This kind of continuous overexposure is not relevant to humans.
Source: FMC Corporation Manchester

(44)

Species: rat **Sex:** female
Strain: Fischer 344
Route of admin.: oral feed
Exposure period: 32 weeks
Frequency of treatment: continuous
Post. obs. period: none
Doses: 15% in diet
Result:
Control Group: yes, concurrent no treatment
Method:
Year: 1978 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Groups of 29 weanling rats (5 weeks of age) were put on either a semipurified diet without carrageenan or one containing 15% carrageenan. At 7 weeks of age the rats in both groups received intrarespiratory instillations of methylnitrosourea (MNU), a known tumor inducer, of 2 mg/rat twice a week for 3 weeks. Controls from each dietary group received an equal volume of phosphate buffer or 0.9% NaCl solution. Body weights of all rats were recorded throughout their life. Thirty weeks after the first instillation the MNU treated groups were necropsied. Controls were continued for another 10 weeks before necropsying (to correspond with other groups receiving treatment with AOM). All organs including the intestines, were examined grossly and histologically for the number and types of tumors.

Results: The incidence and multiplicity of MNU-induced colon tumors in rats fed the carrageenan diet was higher than those in rats fed control diet. Tumors were localized in the distal part of the colon within 4 cm from the anus and in the rats fed a control diet within 7 cm from the anus. The size of colon tumors in rats was also larger with the carrageenan than the control diet.

Conclusion: The rats fed a diet containing carrageenan were more susceptible to colon tumor induction by MNU than were the rats fed a diet without carrageenan. The relevance of this study to human exposure is questionable.
Source: FMC Corporation Manchester

(19)

Species: mouse **Sex:** male/female
Strain:
Route of admin.: oral feed
Exposure period: 24 months
Frequency of treatment: continuous
Post. obs. period: none
Doses: 1, 5, 15, and 25% of diet
Result:
Control Group: yes, concurrent no treatment
Method:
Year: 1959 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Five groups of mice consisting of 5 mice/sex each were administered carrageenan in the diet at 0, 1, 5, 15, and 25% for up to 24 months. All surviving mice after 24 months were given a complete necropsy and organs were examined histologically for signs of disease and carrageenan.

Results: There were no effects that could be attributed to carrageenan consumption seen in this study.
Source: FMC Corporation Manchester

(43)

Species: hamster **Sex:** male/female
Strain: other: Syrian golden
Route of admin.: oral feed
Exposure period: lifespan
Frequency of treatment: continuous
Post. obs. period: none
Doses: 0.5, 2.5, 5.0%
Result:
Control Group: yes, concurrent no treatment
Method:
Year: 1980 **GLP:** no data
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Three groups containing 30 hamsters/sex each received the carrageenan (kappa) in the diet at 0.5, 2.5, and 5.0% for up to 150 weeks or until all rats died or were sacrificed moribund. A control group containing 100 rats/sex was administered untreated diet. Food consumption was measured throughout the study and at death or sacrifice all visceral organs and visible tumors were processed and examined microscopically. All segments of the gastrointestinal tract were carefully examined for signs of toxicity.

Results: There were no significant differences in the survival rates between the experimental and control groups. Animals given the highest dosage level had soft stools during the initial weeks of the test. the average daily intake of the compound was 3719 mg/kg (5%), 2162 mg/kg (2.5%) and 370 mg/kg (0.5%). No histological alterations,

either ulcerative or neoplastic, were found in any groups of treated animals that could be directly related to the administration of the carrageenan.

Source: FMC Corporation Manchester

(42)

5.8 Toxicity to Reproduction

Type: other: Premating to 90 days after birth of F1 offspring
Species: rat **Sex:** male/female
Strain: Sprague–Dawley
Route of admin.: oral feed
Exposure Period: from 14 days prior to mating, through 1–14 days of mating (males sacrificed), through to 90 days after birth of F1.
Frequency of treatment: continuous
Premating Exposure Period
male: 14 days
female: 14 days
Duration of test: until young were 90 days old
Doses: 1.8, 0.9, 0.45% in diet
Control Group: yes, concurrent no treatment
NOAEL Parental: = 1.8 %
NOAEL F1 Offspr.: = 1.8 %
Method:
Year: 1979 **GLP:** no data
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Groups of rats received calcium carrageenan in the diet at concentrations of 0, 0.45%, 0.9% and 1.8%. These diets were available for 14 days prior to mating, throughout the 1–14 days of mating, throughout gestation and until the pups were 90 days old. The F0 males were sacrificed without necropsy after mating was completed. Enough rats were mated in each group to assure the availability of at least 18 litters. Observations were made on reproduction and on the physical and behavioral development of the offspring. All rats were weighed at weekly intervals throughout the test except during breeding. Food consumption was measured on selected breeding pairs, dams, and their offspring in order to determine the amount of food additive each treatment group received. Developmental measures in the offspring included physical development (pinna detachment, incisor eruption, eye opening, and growth), neurohistology and behavior (including surface righting, cliff avoidance, swimming development, pivoting activity, auditory startle, visual placing, rotorod, and others). Litters were subdivided for testing in order to reduce the possibility that experience in one task might alter responses on a subsequent task. Some tests were performed perinatal, others postweaning. At 90 days of age some of the males were sacrificed and the eyes, cerebellum, brainstem, and cerebrum were weighed. A small section of somatosensory cortex was removed, embedded in celloidin for silver impregnation, sliced and examined microscopically.

Results: No dose related changes in any parameter measured

were observed in any of the calcium carrageenan treated groups as compared to the controls. Therefore, continuous exposure to calcium carrageenan at dietary concentrations up to 1.8% has no effect upon the dams or offspring in the protocol described above.

Source: FMC Corporation Manchester

(45)

Type: other: three generation study
Species: rat **Sex:** male/female
Strain: Osborne-Mendel
Route of admin.: oral feed
Exposure Period: from weaning throughout study
Frequency of treatment: continuous
Premating Exposure Period
male: 12 weeks
female: 12 weeks
Duration of test:
Doses: 0.5, 1.0, 2.5 or 5% in diet
Control Group: yes, concurrent no treatment
Method:
Year: 1977 **GLP:** no
Test substance: as prescribed by 1.1 - 1.4
Remark: Methods: Forty males and forty females were used at each dosage level and control. Shortly after weaning all animals were fed carrageenan in the diets at levels of 0.5, 1.0, 2.5, or 5% for 12 weeks before mating. Animals of both F1A and F1B litters were sexed and weighed at birth and weighed also on days 4, 7, 14, and 21. A careful check for abnormalities was made of all rats until weaning at 21 days, when F1A animals were autopsied and discarded. Randomly selected F1B and F2B animals were mated to produce the F2 and F3 generations, respectively. F2A, F2B, F3A and F3B animals were weaned and autopsied in the same manner as F1A animals, except that randomly selected rats from F3A were used for a separate chronic feeding study. On day 20 of pregnancy the F2C and F3C litters were exposed by cesarean section (reported separately). Randomly selected F0, F1B and F2B parental animals were maintained on the same dietary level of carrageenan for 9 months and submitted for pathological examination. Representative weanlings from F2A, F2B, F3A and F3B were also examined for pathology. All adult rats were weighed weekly and food consumption was measured at the same time.

Results: Carrageenan consumption had no effect upon mortality but there was diarrhea was marked in animals fed the two highest dose levels. The cumulative weight gain of the males at week 12 after weaning was significantly reduced at the 5% level in the F0, at the three highest dose levels in the F1B and at all four dose levels in the F2B. Only the females at 5% showed a significant decrease in F2B weight gain. The average daily carrageenan consumption for adult males was 290, 592, 1555, and 3212 mg/kg, and for females was 350, 711, 1832 and 3805 mg/kg. No dose-related difference occurred at any dose level in the fertility

index, but within each generation the fertility was decreased between the first and second litters for each dose level except at 1% in the first generation. There was no indication that the compound had any visible effect on the production of live young. Carrageenan appeared to have no effect on the viability of the young from birth to weaning at day 21; however the weights of the animals at birth appeared to be decreased in the experimental animals, especially at the two highest dose levels. Decreases in weanling weights were dose-related and statistically significant.

In view of the decreased body weights at 1.0, 2.5, and 5.0% dietary levels of carrageenan, the no-effect level in rats is considered to be 0.5%.

Source: FMC Corporation Manchester

(46)

5.9 Developmental Toxicity/Teratogenicity

Species: rat **Sex:** male/female
Strain: Osborne-Mendel
Route of admin.: oral feed
Exposure period: weaning throughout study
Frequency of treatment: continuous
Duration of test: the F2C and F3C generations of a reproduction study were used. Parents were sacrificed gestation day 20.
Doses: 0.5, 1.0, 2.5, 5% in diet
Control Group: yes, concurrent no treatment
NOAEL Maternalt.: = .5 %
NOAEL Teratogen.: = 5 %
Method:
Year: 1977 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Long term multigeneration effects of the dietary intake of calcium carrageenan were evaluated in a three-generation reproduction (males and females dosed) and teratology study. Dietary levels of 0.5, 1.0, 2.5, and 5.0% were ingested throughout the study. The developmental effects were studied in the F2C and F3C litters. One half of the fetuses from each litter at each dose level were examined for skeletal anomalies, while the remaining fetuses were sectioned to detect internal malformations of the soft tissue.

Results: No dose-related effect on body weight gain was observed in mothers of either F2C or F3C litters. The average daily calcium carrageenan consumption of mothers of the F2C animals was 309, 609, 1563, and 3184 mg/kg for the four dose levels respectively, and for the F3C animals was 290, 592, 1524, and 3060 mg/kg. No specific external abnormality nor soft-tissue anomaly was correlated with treatment. No specific skeletal variation could be correlated with dosage either. Both male and female fetuses of treated groups were generally heavier and longer than the

control animals. These increases appear to reflect a chance occurrence since there is no apparent experimental cause for them.

Conclusion: There was no indication that calcium carrageenan was teratogenic in this study.

Source: FMC Corporation Manchester

(47)

Species: rat **Sex:** female
Strain: Wistar
Route of admin.: oral feed
Exposure period: 10 days
Frequency of treatment: continuous
Duration of test: 20 days
Doses: 1% and 5% of diet
Control Group: yes, concurrent no treatment
NOAEL Maternalt.: = 5 %
NOAEL Teratogen.: = 5 %
Method:
Year: 1973 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Twenty pregnant dams were administered the test substance in the diet at concentrations of 1 or 5% from day 6–16 of gestation. Another group of untreated control animals were also included in the study. On day 20 dams were sacrificed and fetuses examined for soft tissue and skeletal abnormalities.

Results: No effects were observed on any parameter examined in this study. Therefore calcium carrageenan is not teratogenic in this model.

Source: FMC Corporation Manchester

(48)

Species: rat **Sex:** female
Strain: Wistar
Route of admin.: oral feed
Exposure period: 10 days
Frequency of treatment: continuous
Duration of test: 20 days
Doses: 1 and 5% of diet
Control Group: yes, concurrent no treatment
NOAEL Maternalt.: = 5 %
NOAEL Teratogen.: = 5 %
Method:
Year: 1973 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Twenty female rats/group were mated and beginning on day 6 of gestation they were fed diets containing either 1 or 5% carrageenan for 10 days. The control group received untreated diet. On day 20 of gestation the dams were sacrificed and the pups were examined for abnormalities. Indices were recorded and the data was analyzed for significant differences.

Results: No differences were detected between the treated and untreated groups. Therefore carrageenan was not considered to be teratogenic in the rat.

Source: FMC Corporation Manchester

(48)

Species: hamster **Sex:** female
Strain: other: golden syrian
Route of admin.: oral feed
Exposure period: 6 days
Frequency of treatment: continuous
Duration of test: 14 days
Doses: 1 and 5% in diet
Control Group: yes, concurrent no treatment
NOAEL Maternalt.: = 5 %
NOAEL Teratogen.: = 5 %
Method:
Year: 1973 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Twenty pregnant hampsters/group were administered the test article in the diet at concentrations of 1 and 5% from day 6 to 11 of gestation. On day 14 the dams were sacrificed and uteri examined for implantation sites; fetuses were removed and examined for signs of soft tissue or skeletal abnormalities.

Results: Carrageenan was not a developmental toxicant in the hamster model.

Source: FMC Corporation Manchester

(48)

Species: hamster **Sex:** female
Strain: other: golden syrian
Route of admin.: oral feed
Exposure period: 6 days
Frequency of treatment: continuous
Duration of test: 14 days
Doses: 1 and 5% in diet
Control Group: no
NOAEL Maternalt.: = 1 %
NOAEL Teratogen.: = 5 %
Method:
Year: 1973 **GLP:** no
Test substance: as prescribed by 1.1 – 1.4
Remark: Methods: Twenty pregnant hampsters/group were administered the test substance in the diet at concentrations of 1 and 5% from gestation day 6–11. On day 14 they were sacrificed and the uteri were examined for implantation sites and fetuses. Offspring were examined for soft tissue and skeletal abnormalities.

Results: A reduced pregnancy rate was obtained at the 5% dosage level. The percent pregnant of the number mated was 68%, compared to 93% in the group receiving 1%, and 82% in the sham–controls. There were no other effects observed on

the developmental criteria examined in this study. Therefore, calcium carragheenan ingestion at high levels may have an effect on the implantation process in this model. It is not a developmental toxin, however, in hamsters.

Source: FMC Corporation Manchester

(48)

5.10 Other Relevant Information

Type: Immunotoxicity

Remark: Methods: In a series of replicate experiments, weanling male DA rats received either distilled water, 5 or 50 mg/kg carrageenan by gavage for 20 days. Rats were then killed and both single cell and adherent cell suspensions of spleen cells were prepared. These were then used to assess the lymphoproliferative response using T cell mitogens PHA–P or Con A in vitro. A pulse of tritiated thymidine was given 18–24 hours before harvesting of cultures, and liquid scintillation counting was performed.

Results: A significant suppression of phytohemagglutinin observed when both the 5 and 50 mg/kg carrageenan–fed groups were contrasted with the distilled water control group. Maximal suppression of the spleen cell response to PHA–P was seen in the 5 mg/kg treatment group, which was significantly different from the 50 mg/kg dose group. There was no significant difference between the non–adherent spleen cell response of distilled water control or carrageenan–fed groups, indicating that the observed suppression of T lymphocyte proliferation was dependent on the presence of adherent cells. Also, when spleen cells from rats receiving carrageenan were cocultured with an equal number of spleen cells from control rats, the control PHA mitogenic response was greatly inhibited. This suggests that active suppression rather than impaired accessory cell function is a requisite for carrageenan–induced abrogation of T cell mitogenesis.

Suppression of spleen cell mitogenesis in response to Con A, on the other hand, was solely observed in rats treated with 5 mg/kg carageenan. The higher dosage level (50 mg/kg) responded like the controls.

Test Substance: As prescribed in 1.1 – 1.4

Source: FMC Corporation Manchester

(49)

Type: Immunotoxicity

Remark: Methods: Male Dark agouti Ag–B4 rats received either distilled water, 5 or 50 mg/kg/day carrageenan via gavage 5 days per week for 4 weeks. Peritoneal exudate cells (PEC) were obtained from at least two rats injected i.p. with corn oil 4 days previously.

Results: PEC macrophage culture supernatants from both dose groups of rats fed carrageenan, suppressed the

normal spleen cell response to PHA. Supernatants from the control group rats also diminished normal spleen cell mitogenesis, but the magnitude of suppression appeared to be much weaker than that from the dosed rats.

Test Material: as prescribed by 1.1 – 1.4

Source: FMC Corporation Manchester

(49)

Type: Immunotoxicity

Remark: Methods: Dark agouti Ag-B4 rats (male) were administered either distilled water, or 5 or 50 mg/kg/day (3 rats/dosage group) carrageenan via gavage 5 days per week for 4 weeks. They were then inoculated with 3.2x10^4 CFU Listeria monocytogenes bacteria into the peritoneum. Eighteen hours later the peritoneal cavity was lavaged with 30 ml sterile PBS. The lavaged material was plated and colonies counted after incubations.

Results: No colonies were observed in liquid from either the control group lavage or the group receiving 50 mg/kg carrageenan. The rats which received 5 mg/kg carrageenan however showed a significant increase in CFUs above the control. Thus early phagocytic resistance to Listeria was diminished in the low dosage group. Carrageenan ingestion may impair intracellular killing or may delay bacterial uptake by phagocytic cells.

Source: FMC Corporation Manchester

(49)

Type: Immunotoxicity

Remark: Methods: Groups of Brown-Norway rats were treated orally with ovalbumin with or without carrageenan by gavage. One group received carrageenan dissolved in saline to form a gel and another group received carrageenan in buffer to avoid gel formation. Blood samples were taken 16 days after priming and evaluated for reaginic response (passive cutaneous anaphylaxis response--PCA). They were then challenged on day 28 with an i.p. injection of OA precipitated with alum and blood was taken again 6 days and 16 days later for the same analysis.

Results: Oral administration of OA induced an immune reaginic tolerance as shown by the absence of antibody production following intraperitoneal immunization with OA and alum. When OA was given with carrageenan in solution, oral tolerance was induced as in the control groups. When carrageenan mixed with OA formed a gel, tolerance was not established. This phenomenon was not caused by oral priming, since the absence of a reaginic response 6 days after immunization was observed, but probably by the retention of protein in the gut lumen.

Carrageenan did not have adjuvant effects when administered orally.

Test substance: iota carrageenan from Mero-Rousselot-Satia

SA
Source: FMC Corporation Manchester

(50)

Type: Immunotoxicity
Remark: Methods: Groups of rats were maintained on tap water or tap water containing 0.25% carrageenan. On day 184 of treatment all rats were challenged intraperitoneally with sheep red blood cells. Serum was collected 7, 14, 21 and 28 days later and analyzed for anti-SRBC antibody activity by the hemagglutination methods.

Results: Animals maintained on carrageenan prior to parenteral challenge with test antigen presented both a delayed and significantly reduced antibody response as compared to the control.

Test Material: no data
Source: FMC Corporation Manchester

(51)

Type: Immunotoxicity
Remark: Methods: PVG strain male rats were randomized into groups and given normal drinking water (control) or drinking water containing 0.5% lambda, or iota, or kappa carrageenan. In each group half the rats were sensitized to sheep red blood cells by i.p. injection on days 55 and 62 of study. All groups received an oral challenge of SRBCs on days 76 and 83. Blood samples for serum-antibody analysis were taken on days 62, 69, 76, 83, and 90. Bile samples for antibody analysis were obtained on day 90 by bile duct cannulation, and animals were killed. Peyer's patches were taken from the proximal, middle and terminal regions of the small intestine and sectioned, stained with H & E (half) or Alcian blue (half-for carrageenan staining). Total IgA content of bile samples was determined. Serum and bile samples were examined for the presence of antibodies with binding specificity for carrageenan. The immune response to the gut commensal E. coli was determined using formalin-fixed organisms in place of SRBCs.

Results: Rats maintained on carrageenan containing water showed no signs of abnormal behaviour or appearance. Body weight gain and food and water consumption were similar to the controls'. Sections of intestines and Peyer's patches examined microscopically revealed no histological abnormalities or pathological lesions attributable to treatment. However the Alcian blue stain specific for carrageenan revealed small numbers of cells positive for Alcian blue within the intestinal villi, lamina propria and basement-membrane lymphatics of treated rats. The type of carrageenan had no apparent effect on the degree of uptake or the distribution within the intestinal tissues.

The feeding regimens did not modify either the rate of bile secretion or the total IgA concentration in the bile. Serum and bile samples taken at 90 days showed an absence of

antibodies indicating that although carrageenans gained access to lymphoid tissues, a specific anti-carrageenan antibody response could not be demonstrated. Antibody analysis of 90 day bile samples for humoral reactivity to intestinal bacteria demonstrated antibodies in equivalent amounts in all test and control samples. Examination of day 90 samples of serum for antibacterial antibody activity revealed that all animals previously sensitized to SRBCs possessed higher levels of anti-E. coli antibodies than the nonsensitized groups. A similar picture emerged when the same serum samples were analyzed for antibodies with binding specificity for total gut bacteria using the indirect immunofluorescence assay, but since equivalent levels of antibody were detected in pre-sensitized control rats, the immunization effect is clearly unrelated to the feeding regimen and apparently relates to the parenteral administration of SRBCs.

Source: FMC Corporation Manchester

(52)

Type: Immunotoxicity

Remark: Methods: The graft-versus-host popliteal lymph node assay was used to assess the effect of orally administered carrageenans on T cell reactivity. Rats were exposed to iota, kappa, or lambda carrageenan in the drinking water at 0.5% continuously for 90 days. In addition other rats were given drinking water containing 0.5% iota carrageenan or 0.5% degraded iota carrageenan. After 90 days, the rats were killed and cell suspensions were prepared in PBS from the spleens and mesenteric lymph nodes of the control and exposed animals. Suspensions from test or control donors were injected s.c. into the right hind footpad of hybrid recipients (PVG x A) matched for sex and age. The left footpad was injected with (PVG x DA) F1 (self) cells to serve as a negative control. Seven days after injection rats were killed and the right and left popliteal lymph nodes were removed, and weighed. The proliferation index was calculated as the comparison of the weight of the test lymph node to that of the control.

Results: The proliferation index was similar across all groups. Therefore carrageenan does not affect T cell competence.

Source: FMC Corporation Manchester

(52)

Type: Immunotoxicity

Remark: The in vivo relevance of the observed in vitro suppression of T lymphocyte proliferation by low doses of carrageenan was examined by administering carrageenan orally to rats according to 2 different protocols. In the initial experiments, a single dose of 0.5 to 50 mg carrageenan was instilled directly into the stomach of Lewis rats via a gastric tube. The effect of carrageenan ingestion on the proliferative response (using tritiated thymidine incorporation) of spleen and lymph node cells in response to phytohemagglutinin 3 days after administration was in

agreement with in vitro observations--low doses of carrageenan resulted in significant suppression of spleen and mesenteric lymph node responses while high doses showed no effect.

In a second series of experiments, 2 litters of DA rats were weaned on distilled water containing 0, 0.1 or 1.0 mg/ml carrageenan and maintained on this regimen for 8 weeks. Those maintained on 0.1 mg/ml carrageenan showed markedly suppressed spleen cell responses to PHA while those maintained on 1.0 mg/ml gave responses equivalent to the distilled water controls. When glass wool-adherent cells (macrophages) were removed from the spleens of the 0.1 mg/ml group, responsiveness equivalent to control values was restored. Supernatants obtained from peritoneal exudate cells of rats maintained on carrageenan at 0.1 mg/ml were highly suppressive to the PHA response of normal spleen cells. These data support the hypothesis that low doses of carrageenan in vivo as well as in vitro stimulate a population of macrophages which suppress T lymphocyte proliferation via production of a soluble inhibitor.

Source: FMC Corporation Manchester

(53)

Type: other: Absorption and Distribution Study

Remark: Method: Groups of male PVG strain rats received 50 ug tritiated carrageenan (specific activity about 10^7) in 1 mg deionized water. Urine and feces were collected throughout the test. Groups of animals were killed at 6, 24, 48 and 72 hours after dosing and the following tissues were removed for oxidation and scintillation counting; liver, kidney, spleen, mesenteric lymph node, cecal lymph node, Peyer's patches and washed duodenum (free of Peyer's patches).

Results: The majority of the administered radioactivity was excreted in the feces. Some uptake of the label into other tissues was observed; however, the authors caution that due to the exchange of the tritium label with total body water, only levels of radioactivity greater than those found in serum can be considered significant. Thus, only the cecal lymph nodes, Peyer's patches and the intestinal wall exhibited uptake of carrageenan. Examination of selected tissues by autoradiography revealed that the bound tritium was invariably associated with macrophages. This indicates that a very small percentage of orally administered food-grade iota carrageenan can gain access to the body tissues via the Peyer's patches and cecal lymph node.

Source: FMC Corporation Manchester

(54)

5.11 Experience with Human Exposure

Remark: Methods: The National Maternal and Infant Health Survey data was examined to determine whether there was an association between frequency of symptomatic upper respiratory tract infection in the first 6 months of life and the type of formula (liquid containing 0.03% carrageenan or powdered without carrageenan) in full-term babies. Sample consisted of 1269 infants who consumed liquid formula exclusively for the first 6 months and 149 infants who consumed powdered formula exclusively for that period of time. On average it was estimated that during that 6 months of life a child receiving liquid formula would consume 2.64 g carrageenan/day.

Results: A slightly higher (53.4%) proportion of the infants receiving liquid formula were illness-free for the first 6 months of life as compared with those infants receiving powdered formula (47.6%). In looking at the number of months in which at least one upper respiratory infection had occurred, there was a slight advantage for liquid formula; infants receiving liquid formula had an average 0.9 months in which an upper respiratory infection occurred compared with 1.0 months for infants receiving powdered formula.

Similar analysis of 364 preterm infants, 333 of whom received liquid formula, showed the same lack of effect. Therefore the data suggest that carrageenan-containing liquid infant formula is not immunosuppressive.

Test Material: undefined multiple sources

Source: FMC Corporation Manchester

(55)

Remark: Methods: A total of 154 strains from 22 species of anaerobic bacteria present in high concentrations in the human colon were surveyed for their ability to ferment 21 different complex carbohydrates, including carrageenan. Fermentation was determined by using the replicator method of Wilkins and Walker. Trypticase-yeast extract replicatory medium was used as the basal medium. Polysaccharides were autoclaved in distilled water before addition to the medium, and rumen fluid was added when required by the anaerobes being used. Strains were classified as fermenting a substrate if they lowered the pH of the medium by at least 1 pH unit as compared with uninoculated control wells and inoculated wells of the basal medium without carbohydrate.

Results: Carrageenan along with 10 other substrates were not fermented by any of the strains tested.

Source: FMC Corporation Manchester

(56)

Remark: Carrageenan (E 407) has been an approved food additive for more than 50 years worldwide. In the EU, carrageenan is listed in Annex I at Quantum Satis (QS) in the European Parliament and Council Directive No. 95/2/EC of 20 February 1995 on "Food Additives Other Than Colours and Sweeteners". Carrageenan is used primarily as a gelling agent and fat substitute in concentrations of 0.1–0.5% in various foodstuffs. The Acceptable Daily Intake (ADI) currently established in the EU is 0–75 mg/kg bodyweight.

Source: FMC Corporation Manchester

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7.1 Risk Assessment

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WHO FOOD ADDITIVES SERIES: 59

Safety evaluation of certain food additives and contaminants

Prepared by the
Sixty-eighth meeting of the Joint FAO/WHO
Expert Committee on Food Additives (JECFA)

Food and Agriculture Organization of the United Nations

World Health Organization



IPCS

International Programme on Chemical Safety

World Health Organization

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This publication is a contribution to the International Programme on Chemical Safety.

The **International Programme on Chemical Safety (IPCS)**, established in 1980, is a joint venture of the United Nations Environment Programme (UNEP), the International Labour Organization (ILO) and the World Health Organization (WHO). The overall objectives of the IPCS are to establish the scientific basis for assessing the risk to human health and the environment to exposure from chemicals, through international peer review processes as a prerequisite for the promotion of chemical safety, and to provide technical assistance in strengthening national capacities for the sound management of chemicals.

The Inter-Organization Programme for the Sound Management of Chemicals (IOMC) was established in 1995 by UNEP, ILO, the Food and Agriculture Organization of the United Nations, WHO, the United Nations Industrial Development Organization, the United Nations Institute for Training and Research and the Organisation for Economic Co-operation and Development (Participating Organizations), following recommendations made by the 1992 UN Conference on Environment and Development to strengthen cooperation and increase coordination in the field of chemical safety. The purpose of the IOMC is to promote coordination of the policies and activities pursued by the Participating Organizations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

PREFACE

The monographs contained in this volume were prepared at the sixty-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at WHO headquarters in Geneva, Switzerland, on 19–28 June 2007. These monographs summarize the data on selected food additives and contaminants reviewed by the Committee.

The sixty-eighth report of JECFA has been published by the World Health Organization as WHO Technical Report No. 947. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives, the Codex Committee on Contaminants in Food and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and toxicological monographs, such as those contained in this volume, on substances that they have considered.

The monographs contained in the volume are based on working papers that were prepared by temporary advisers. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers. The monographs were edited by M. Sheffer, Ottawa, Canada.

Many unpublished proprietary reports are unreferenced. These were voluntarily submitted to the Committee by various producers of the food additives under review and in many cases represent the only data available on those substances. The temporary advisers based the working papers they wrote on all the data that were submitted, and all these reports were available to the Committee when it made its evaluations.

The preparation and editing of the monographs included in this volume were made possible through the technical and financial contributions of the Participating Organizations of the International Programme on Chemical Safety (IPCS), which supports the activities of JECFA.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the organizations participating in the IPCS concerning the legal status of any country, territory, city or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the organizations in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological or toxicological properties of the compounds evaluated in this publication should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Expert Committee on Food Additives, International Programme on Chemical Safety, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland.

SPECIFIC FOOD ADDITIVES (OTHER THAN FLAVOURING AGENTS)

ACIDIFIED SODIUM CHLORITE

First draft prepared by

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1. EXPLANATION

1.1 Introduction

Acidified sodium chlorite (ASC) possesses antimicrobial properties and is intended for use primarily as a spray or dipping solution for poultry, meats, vegetables, fruits and seafoods. It is also used in poultry chilling water. ASC is produced by the addition of a food-grade acid (e.g. citric acid, phosphoric acid, hydrochloric acid, malic acid or sodium hydrogen sulfate) to an aqueous solution of sodium chlorite. Combining the acid with sodium chlorite results in conversion of chlorite to chlorous acid, which can subsequently form a mixture of chlorite, chlorate, chlorine dioxide and chloride. The Committee has not previously evaluated ASC or sodium chlorite used in the preparation of ASC. Among the acids mentioned above that may be used in the preparation of ASC, sodium hydrogen sulfate has not been evaluated by the Committee.

ASC was evaluated by the Committee at its present meeting as requested by the Codex Committee on Food Additives and Contaminants at its thirty-eighth session (Codex Alimentarius Commission, 2006). The Committee was asked to provide a risk assessment for ASC for use in food contact (as a processing aid).

The safety of chlorite, chlorate, chlorine dioxide and chloride in drinking-water has previously been assessed by the World Health Organization (WHO). WHO established tolerable daily intakes (TDIs) of 0.03 mg/kg body weight (bw) for both chlorite and chlorate (World Health Organization, 2003, 2004).

Residual chlorine dioxide is lost by evaporation; hence, chlorite, chlorate and chloride are the principal residues expected. The chloride generated as a result of treatment with ASC is negligible compared with the chloride already present in food. The use of chlorine to disinfect water supplies results in formation of by-products such as trihalomethanes. However, chlorine dioxide acts as an oxidizing agent and therefore does not form trihalomethanes or by-products other than chlorite and chlorate ions. The residues of the food-grade acids (e.g. phosphate, citrate, malate, sulfate) are commonly present in food and have previously established acceptable daily intakes (ADIs). Therefore, the Committee focused the toxicological evaluation on ASC, chlorite and chlorate.

The Committee received a submission containing published information on ASC, including studies on a germicidal product developed for clinical uses and on sodium chlorite. Additional information identified by a literature search relates to the safety, absorption, distribution, metabolism and excretion or biochemistry of ASC, chlorite or chlorate in relation to animal, human or in vitro models.

1.2 Compounds considered in the toxicological evaluation

The germicidal ASC product described in the submission is formed by combining sodium chlorite with lactic acid and is available in liquid or gel forms. Each form comes in two parts that are mixed together in equal quantities immediately prior to use. In the liquid form, the first part (part A) contains 79% sodium chlorite, with the remainder being tetrasodium ethylenediaminetetraacetic acid (EDTA). Part B contains 88% lactic acid, with the remainder being a non-ionic surfactant (pluronic F-68) and water. On mixing parts A and B, chlorine dioxide is released. The gel form contains the same ingredients and a gelling agent (magnesium aluminium silicate). Hence, the sodium chlorite content of both the liquid and the gel is 395 mg/g, or 294 mg/g expressed as chlorite (ClO_2^-).

1.3 Chemical and technical considerations

Sodium chlorite is marketed in two forms, as a solid containing approximately 80% sodium chlorite and as an aqueous solution. Sodium chlorite is manufactured by reducing sodium chlorate, chemically or electrochemically, in the presence of hydrochloric acid to produce chlorine dioxide. Chlorine dioxide is then reacted with hydrogen peroxide in aqueous sodium hydroxide to yield a solution containing 30–50% sodium chlorite, which is subsequently dried to a solid or further diluted to get an aqueous solution.

ASC is intended for use as part of an integrated approach designed to control microbial loads on foodstuffs. ASC solution acts to reduce the number of pathogens (e.g. *Escherichia coli* O157:H7, *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes*), as well as, to a somewhat lesser extent, spoilage bacteria found

on the surface of foods during processing. The solution is applied onto the surface of different types of fresh and processed foods at a concentration range of 50–1200 mg sodium chlorite/l. Fresh and processed fruits and vegetables are subjected to a water rinse after ASC application followed by a 24-h withholding time (for cut produce only). Treatment of whole or parts of poultry carcasses, sausages or delicatessen meats (cold cuts) is carried out by spraying or dipping prior to or after chilling. ASC is also used to treat pre-chilling and chilling water, into which poultry carcasses are submerged, at relatively low levels (i.e. 50–150 mg sodium chlorite/l). Poultry and meat products are not rinsed subsequent to treatment.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion

(a) Acidified sodium chlorite

Groups of 3–5 female Sprague-Dawley rats (weight range 200–300 g) were administered 3 ml of a germicidal ASC liquid containing 19.05 mg $^{36}ClO_2^-$ once by gavage (average dose about 70 mg $^{36}ClO_2^-$/kg bw). Separate groups were used for determination of the ^{36}Cl content of blood and tissue samples, protein binding and excretion over 144 h. The maximal plasma concentration of ^{36}Cl occurred at 8 h, and the half-life of ^{36}Cl elimination from plasma was 48 h. About 20–30% of the radiolabel appeared to be bound to protein in liver, plasma and red blood cells. At 144 h, the concentration of ^{36}Cl was highest in plasma, followed by lung, kidney, skin, bone marrow, stomach, ovary, duodenum, ileum, spleen, fat, brain, liver and carcass. Within this time, 45% of the dose was eliminated in the urine and 10% in faeces. ^{36}Cl was not detected in expired air. Chloride and chlorite, but not chlorate, were detected in rat urine (Scatina et al., 1983).

The toxicokinetics of a germicidal ASC gel have been investigated following dermal application. The gel was applied to the shaven backs of groups of 4 or 10 female Sprague-Dawley rats (270–290 g) at a dose of 2 g gel/kg bw daily for 10 days (equivalent to 588 mg chlorite/kg bw per day) in order to reach a plateau. On the 11th day, the ^{36}Cl-labelled gel was applied at a dose of 2 g/kg bw and covered to prevent ingestion. Separate groups were used for determination of the ^{36}Cl content of blood and tissue samples, protein binding and excretion over 168 h. One control animal did not receive any gel or other treatment. The peak plasma concentration of ^{36}Cl was observed after 72 h, and the half-life for ^{36}Cl elimination from plasma was 64 h. The highest concentrations of radioactivity were found in whole blood, followed by kidney, plasma, untreated skin, ovary, lung, ileum, spleen, brain, duodenum, liver, stomach, bone marrow, carcass and fat. Urinary excretion of radioactivity was greatest in the first 24 h. Radioactivity was not detected in faeces or expired air at any time point. The authors concluded that topical application of the gel resulted in a much slower rate of absorption and an increased half-life for elimination of ^{36}Cl compared with oral administration of the liquid germicidal product (Scatina et al., 1984).

(b) Sodium chlorite and chlorate

^{36}Cl compounds were administered by gavage to groups of four male Sprague-Dawley rats at the following doses: 0.15 mg chlorite/kg bw, 3.26 mg hypochlorous acid/kg bw, 1.5 mg chlorine dioxide/kg bw and 0.065 mg chlorate/kg bw. Separate groups were used for determination of the ^{36}Cl content of blood and tissue samples, protein binding and excretion over 72 h. The time taken to absorb 50% of the dose for each chlorine species was found to be longest for hypochlorous acid (4.42 ± 1.31 h), followed by chlorite (3.5 ± 1.06 h), chlorate (1.74 ± 0.66 h) and chlorine dioxide (0.18 ± 0.01 h). The time taken to eliminate 50% of the dose from the plasma when detected as ^{36}Cl was longest for hypochlorous acid (77.0 ± 8.8 h), followed by chlorine dioxide (43.9 ± 2.3 h), chlorate (36.7 ± 5.8 h) and chlorite (35.2 ± 3.0 h). After 72 h, radioactivity from chlorite was found at the highest level in the plasma, followed by stomach, testes, skin, lung, duodenum, kidney, carcass, spleen, ileum, bone marrow and liver. Radioactivity from hypochlorous acid was highest in plasma, followed by bone marrow, kidney, testes, lung, skin, duodenum, spleen, stomach, liver, carcass and ileum. Radioactivity from chlorine dioxide was highest in the kidney, followed by lung, plasma, stomach, ileum, liver, duodenum, spleen and bone marrow. Radioactivity from chlorate was highest in plasma, followed by stomach, lung, testes, kidney, skin, duodenum, spleen, ileum, carcass, liver and bone marrow. In blood, chlorite levels were distributed evenly between plasma and packed cells, whereas chlorate preferentially accumulated in the plasma.

The percentage of the initial dose excreted within 72 h ranged from 28% for hypochlorous acid to 43% for chlorate, with 75–87% present in the urine and the remainder in the faeces. No ^{36}Cl was detected in expired air (Abdel-Rahman et al., 1982a).

2.1.2 Biotransformation

In the studies of the germicidal ASC product described in section 1.2 above, chloride and chlorite, but not chlorate, were detected in rat urine. Between 8 and 72 h following oral administration, the chloride and chlorite were present at equal concentrations; however, during the first 8 h and between 120 and 144 h, chloride was excreted to a greater extent than chlorite. Following dermal administration, the total excretion of chloride and chlorite was approximately the same. The authors reported these as metabolites of chlorine dioxide (Scatina et al., 1983).

In the studies of Abdel-Rahman et al. (1982a), described in section 2.1.1, chloride, chlorite and chlorate were found in rat urine after administration of chlorine dioxide, chlorite and chlorate. The major metabolite in all cases was chloride, representing 26.9% of the initial dose of chlorine dioxide, 31.6% of the initial dose of chlorite and 20.5% of the initial dose of chlorate (Abdel-Rahman et al., 1982a).

In the above studies, it is unclear if the substances described as “metabolites” resulted from degradation of the administered substances prior to absorption or are biotransformation products within the body.

The formation of chloroform was investigated in groups of 4–9 male Sprague-Dawley rats that received ^{36}Cl-labelled chlorine dioxide, chlorite or chlorate at 10 or 100 mg/l in drinking-water for 20 h/day, 7 days/week, for 12 months. The authors noted that administration of chlorine dioxide at 100 mg/l increased chloroform levels in the blood, liver, testes and brain, whereas at 10 mg chlorine dioxide/l, chloroform levels were increased in the testes only. Chlorite at 100 mg/l elevated chloroform levels in the liver and brain but not in the blood. Chlorate increased chloroform levels in the liver but not in the blood. Details of the amounts of chloroform generated were not provided (Abdel-Rahman et al., 1982a).

2.1.3 Effects on enzymes and other biochemical parameters

A role of sodium chlorite in supporting cytochrome P450–catalysed steroid hydroxylation was studied using microsomal fractions prepared from the livers of male Sprague-Dawley rats. In the presence of 4 mmol sodium chlorite/l, the generation of hydroxylated androstenedione metabolites during a 10-min incubation period was 67% of that in the presence of 1 mmol of reduced nicotinamide adenine dinucleotide phosphate (NADPH) per litre (Hrycay et al., 1975).

Blood from male CD rats and healthy human volunteers was used in a study designed to demonstrate the ability of sodium chlorite to react with constituents of the erythrocyte in vitro. Incubation for 1 h with 10^{-4} and 10^{-1} mol sodium chlorite/l resulted in a concentration-related increase in methaemoglobin and hydrogen peroxide levels and a decrease in glutathione (GSH) levels (Heffernan et al., 1979a).

To study the effects of the germicidal ASC liquid (described in section 1.2) on blood GSH, erythrocyte osmotic fragility and the GSH-dependent enzyme system in vitro, whole blood from male Sprague-Dawley rats was incubated with the germicidal ASC liquid (with a final sodium chlorite concentration of 1 or 2 mmol/l) for 0.5, 1, 2 and 4 h. Methaemoglobin was measured after 2 h of incubation. A concentration-related decrease in GSH content was seen, with the maximal effect occurring after 30 min of incubation. Rat blood osmotic fragility was significantly decreased at both test concentrations after 60 and 120 min of incubation compared with controls. Methaemoglobin was detected at a low concentration in samples after 2 h of treatment at 2 mmol sodium chlorite/l but not at 1 mmol sodium chlorite/l. Addition of reduced GSH to the rat blood immediately prior to addition of the germicidal ASC liquid or of GSH reductase and NADPH immediately after the addition of the germicidal ASC liquid returned the osmotic fragility to levels close to those of the control. After incubation of whole blood with the germicidal ASC liquid containing up to 2 mmol sodium chlorite/l for 1 h, GSH reductase and GSH peroxidase activities were unchanged. The authors concluded that this ASC product probably damages the erythrocyte membrane (Abdel-Rahman & Scatina, 1985).

Sodium chlorite, sodium hypochlorite and sodium chlorate were used in a number of in vitro tests to identify their effects on erythrocytes, fibroblasts and endothelial cells. Sodium chlorite (≥0.04 mmol/l) increased the levels of methaemoglobin in the erythrocytes. Sodium chlorite solution (0.2 mmol/l) caused fibroblast cells to become misshapen and swollen, and 1 mmol sodium chlorite/l resulted in endothelial cell death (Habermann & Müller, 1989).

Methaemoglobin formation was measured in erythrocytes from Dorset sheep incubated with 1, 2, 3 or 4 mmol sodium chlorite/l. There was a concentration-dependent increase in methaemoglobin formation. The potency of sodium chlorite was much less than that for other agents such as *p*-dinitrobenzene and nitrite, but much greater than that for chlorate (concentration expected to induce 15% methaemoglobin: *p*-dinitrobenzene = 0.01 mmol/l, nitrite = 0.79 mmol/l, chlorite = 2.68 mmol/l and chlorate = 22.45 mmol/l) (French et al., 1995).

Sodium chlorite and chlorate did not induce lipid peroxidation at concentrations up to 400 µmol/l in an in vitro system of multilayer phospholipid liposomes prepared from egg phosphatidylcholine (Panasenko et al., 1997).

The effects of sodium chlorite on membrane components and antioxidant depletion have been studied in rabbit corneal epithelial cells, human conjunctival epithelial cells, phospholipid vesicles prepared from egg yolk and GSH in solution. Incubation of phospholipid vesicles with 3.5 mmol sodium chlorite/l for up to 2 h had no effect, whereas incubation for 48 h resulted in lipid depletion and an increase in lipid oxidation. Sodium chlorite was found to be a very potent GSH oxidizing agent: at a GSH/sodium chlorite ratio of 0.5, GSH was depleted after 5 min. GSH depletion was also seen in rabbit corneal epithelial cells and human conjunctival epithelial cells incubated with 3.5 mmol sodium chlorite/l or 0.55 mmol sodium chlorite/l. At 3.5 mmol/l, sodium chlorite caused rapid loss of cell viability in the corneal cells, as assessed by trypan blue staining and loss of adherence. At 0.55 mmol/l, sodium chlorite had very little effect over the first few hours but decreased viability after 24 h. The conjunctival cells appeared to be less sensitive than the corneal cells. No oxidatively modified lipids could be detected in the cells following sodium chlorite treatment, and no effects were seen in levels of cytosolic antioxidants (Ingram et al., 2003).

In a subsequent study, rabbit corneal epithelial and human conjunctival epithelial cells were incubated with 0, 5, 25, 50, 100, 250, 500 or 750 mg sodium chlorite/l. Depletion of cellular adenosine triphosphate (ATP) occurred at concentrations above 100 mg/l for rabbit corneal epithelial cells and above 50 mg/l for human conjunctival epithelial cells. GSH was depleted progressively between 5 and 250 mg/l in both cell types. The authors concluded that sodium chlorite is of low toxicity to ocular cells (Ingram et al., 2004).

2.2 Toxicological studies

2.2.1 Acute toxicity

The available LD_{50} data do not indicate marked differences in susceptibility between species (Tables 1–3).

Studies in rabbits show that the ASC germicidal gel product and/or sodium chlorite have the potential to cause skin and eye irritation (Abdel-Rahman et al., 1982b; Seta et al., 1991). A sensitization test of the germicidal liquid and gel in guinea-pigs involved intradermal injection of an initial dose of 50 mg followed by nine injections of 100 mg of each test substance over a period of 3 weeks. No sensitization effects were seen. Necrosis was seen following injection of the

Table 1. Acute toxicity of ASC

Species	Sex	Route (form)	LD_{50} (mg chlorite/kg bw)	Reference
Rat	Male	Oral (liquid germicidal ASC product)	292[a]	Abdel-Rahman et al. (1982b)
Rat	Female	Oral (liquid germicidal ASC product)	340[a]	Abdel-Rahman et al. (1982b)
Rabbit	NS	Dermal (gel germicidal ASC product)	>422	Abdel-Rahman et al. (1982b)

NS, not stated.
[a] A 14-day observation period.

Table 2. Acute toxicity of sodium chlorite

Species	Sex	Route	LD_{50} (mg/kg bw)[a]	Reference
Mouse	NS	Oral	350 (267–433)	Pisko et al. (1980)
Rat	Male	Oral	158	Seta et al. (1991)
Rat	Female	Oral	177	Seta et al. (1991)
Rat	NS	Oral	350 (251–449)	Pisko et al. (1980)
Rat	NS	Oral	165	Perry et al. (1994)
Rat	NS	Oral	105	World Health Organization (2003)
Guinea-pig	NS	Oral	300	Pisko et al. (1980)
Quail	NS	Oral	496	World Health Organization (2003)

NS, not stated.
[a] Unclear if doses expressed as sodium chlorite or chlorite. Mean provided; numbers in parentheses are ranges.

germicidal liquid, which the authors attributed to the pH of the test substance, based on the similarity to the response observed with citric acid solution at the same pH (2.9) (Abdel-Rahman et al., 1982b).

Table 3. Acute toxicity of sodium chlorate

Species	Sex	Route	LD_{50} (mg/kg bw)	Reference
Dog	NS	Oral	600[a]	Sheahan et al. (1971)

NS, not stated.
[a] Unclear if doses expressed as sodium chlorate or chlorate.

2.2.2 Short-term studies of toxicity

(a) Acidified sodium chlorite

Guinea-pigs

The toxicity of the germicidal gel and liquid ASC products has been investigated following vaginal administration to female albino Hartley guinea-pigs. Groups of eight animals received the germicidal gel at a dose of 1 g/kg bw, a high concentration of the liquid at a dose of 2.5 g/kg bw or a low concentration of the liquid (60% of the high concentration), equivalent to 294, 735 and 441 mg chlorite/kg bw. The gel was administered intravaginally daily for 30 days, whereas the liquid was applied over the external vaginal area 3 times per day for 10 days. Observations included overt signs of toxicity, food consumption, body weights, blood and urinalysis, ophthalmological examinations, macroscopic abnormalities at necropsy and histopathology. The only observations significantly different in the germicidal product–treated animals compared with placebo were as follows. Haemoglobin, mean cell haemoglobin and direct bilirubin were increased and carbon dioxide was decreased in the blood of the high- and low-concentration liquid groups: all were considered to be within the normal range. A significant decrease in the relative liver weight was reported for the germicidal gel group. Some changes in relative organ weights were reported for the animals receiving the germicidal liquid, although these were mostly not concentration related and were not accompanied by histological changes. Fatty livers were detected in two animals from each group in the gel study, including placebo and saline control, and therefore were not considered related to the germicidal product. Macroscopic and histological changes were observed in the vaginas of animals treated with the germicidal gel but not the liquid (Abdel-Rahman et al., 1987b).

Rabbits

The germicidal gel product described in section 1.2 was applied to the shaven skin of albino New Zealand White rabbits (13 of each sex) at 0.5, 1.0 or 2.0 g/kg bw (equivalent to 147, 294 and 588 mg chlorite/kg bw per day) daily for 4 weeks. A control group received the gel base with no active ingredients. Blood samples were taken after 15 days and at the end of the treatment and analysed for a range of parameters. There were no overt signs of toxicity or differences in weight gain or relative organ weights. The haematocrit percentage was significantly increased and mean corpuscular haemoglobin concentration significantly decreased in the group

receiving 2.0 g gel/kg bw. There was an apparently dose-related decrease in blood albumin, which was statistically significant in the 2.0 g/kg bw group. Other reported significant changes were increases in creatinine and indirect bilirubin and a decrease in direct bilirubin at 1.0 and/or 2.0 g/kg bw group, which were not dose related (Abdel-Rahman et al., 1982b).

In a subsequent study, groups of six male and six female albino New Zealand rabbits were treated with high- or low-concentration germicidal gel at 2 g/kg bw or with high- or low-concentration germicidal liquid at 3 g/kg bw applied to the shaved, lightly abraded skin 5 days/week for 30 days. The high-concentration gel was 4-fold more concentrated than the low-concentration gel, and the high-concentration liquid was 1.67-fold more concentrated than the low-concentration liquid, but actual doses of chlorite are unclear. Control groups received placebo gel or liquid or were untreated. Observations included food consumption, body weights, clinical signs, blood and urinalysis. At the end of the study, a necropsy was performed, with a number of organs weighed and examined histologically: liver, kidney, spleen, adrenal glands, thymus, pituitary, brain, pancreas, testes, ovary, uterus, application sites and grossly abnormal tissues.

Signs of skin irritation were observed at the site of application from day 3 to the 3rd week of treatment in the high-dose gel group. Milder signs of irritation were apparent in both the low- and high-dose liquid groups, which resolved by day 11. In the three gel groups (including placebo), the ratio of blood urea nitrogen (BUN) to creatinine was significantly reduced compared with controls. In the germicidal liquid groups, BUN, chloride, total protein and albumin were significantly reduced compared with controls. The haematological and clinical chemistry changes were within normal ranges and mostly not dose related. There were some changes in relative organ weights, which were not dose related except for increases in relative ovary and pancreas weights in the germicidal liquid–treated animals; these changes in relative organ weights were not accompanied by histopathological changes. The reported histological abnormalities were mainly inflammatory changes in the skin at the site of application. Increased glycogen content of the liver was seen in 2–3 animals from each liquid-treated group as well as in the control animals (Abdel-Rahman et al., 1987a).

The germicidal gel was applied to the shaven backs of groups of New Zealand albino rabbits (four per sex) 5 days/week for 3 months at doses of 0, 0.5, 1.0 or 2.0 g gel/kg bw (equivalent to 0, 198, 395 and 790 mg sodium chlorite/kg bw per day, or 0, 147, 294 and 588 mg chlorite/kg bw per day). Control groups received either a control gel (with no active ingredients) at 2.0 g/kg bw or no treatment. Food consumption and body weights were recorded. At the end of the dosing period, a necropsy was performed and blood was analysed for haematological parameters, osmotic fragility, methaemoglobin and GSH content. The BUN/creatinine ratio was reduced in the 2.0 g/kg bw germicidal product–treated group compared with controls. Other reported changes occurred either at one of the lower concentrations or also in the placebo gel group (Scatina et al., 1984).

(b) Sodium chlorite

Rats

Male CD weanling rats (six per group) were given reagent-grade sodium chlorite in their drinking-water for 30, 60 or 90 days at concentrations of 100, 250 or 500 mg/l, expressed as chlorite. Water consumption data were not provided for this study. If it is assumed that male rats consume about 25 ml water per day and the available mean body weights for each group are used, the chlorite intake can be estimated to be 7, 17 and 33 mg/kg bw per day at 30 days and 6, 13 and 26 mg/kg bw per day at 60 days for the 100, 250 and 500 mg/l dose groups, respectively. Body weights for the 90-day groups were not provided; however, based on the assumption of a body weight of 500 g, the intakes were estimated to be 5, 13 and 25 mg/kg bw per day. These estimates could be affected by reduced consumption at higher concentrations due to the reduced palatability of the drinking-water containing the test substance. Body weights were recorded at the beginning and end of treatment, and liver, kidney and spleen weights were recorded at necropsy for the 30- and 60-day treatment groups. Haematological parameters were investigated. The authors reported "few signs" of overt toxicity, but an increase in relative kidney weight was observed in animals receiving 500 mg chlorite/l for 60 days. After 30 days of treatment in the high-dose animals, erythrocyte count, haemoglobin and packed cell volume were reduced; after 60 days, a similar but smaller effect was observed; and by 90 days, the animals had increased erythrocyte, haemoglobin and packed cell volume. GSH levels were significantly reduced in the two groups of animals receiving 100 and 250 mg chlorite/l, and this was associated with an increase in hydrogen peroxide levels in erythrocytes. No significant increases in methaemoglobin levels were seen for any of the dose groups (Heffernan et al., 1979b).

In a range-finding study, groups of five male and five female CRL:CD (SD) BR strain rats were administered technical-grade sodium chlorite daily by gavage for 14 days at doses of 0, 25, 50, 100 or 200 mg sodium chlorite/kg bw per day (adjusted for purity), equivalent to 0, 19, 37, 75 and 149 mg chlorite/kg bw per day, respectively. The major impurities in the test material were sodium chloride (9.7%) and water (5.7%), with lesser amounts of sodium chlorate, sodium hydroxide, sodium sulfate and sodium carbonate. Three animals in the top dose group died within 3 days, and the remainder were sacrificed on day 3. There was evidence of methaemoglobinaemia in these animals. There were clear signs of toxicity at 100 mg sodium chlorite/kg bw per day (decreased body weight gain, changes in blood parameters), but not at the lower doses (Harrington et al., 1995a).

This study was used as a basis for selecting doses of 0, 10, 25 or 80 mg sodium chlorite/kg bw per day (adjusted for purity), equivalent to 0, 7.4, 19 and 60 mg chlorite/kg bw per day, respectively, for a 13-week study using the same test material and strain of animals (15 per sex per dose group). The study appears to have been conducted in accordance with the Organisation for Economic Co-operation and Development (OECD) guideline in place at the time, but this is not mentioned in the publication. Animals were weighed weekly throughout the study and subjected to ophthalmological examination before and after dosing. Blood and

urine samples were taken from all animals during week 13 of the study and analysed for a full range of parameters. At sacrifice, all animals were weighed and examined externally, and a gross macroscopic examination was performed. Microscopic examination was carried out on an extensive range of organs and all gross lesions.

At 80 mg/kg bw per day, there were four deaths and adverse effects on blood parameters in surviving animals. In the male animals, dose-related trends were reported for erythrocyte and lymphocyte counts (decreases) and for methaemoglobin and neutrophil counts (increases). At 10 and 25 mg/kg bw per day, mean values of the above-mentioned parameters were reported to be within the background range, and these were not considered to be toxicologically significant. In females, the effects on blood parameters were less marked, and methaemoglobin levels were decreased rather than increased. A dose-related increase in spleen weights was seen in both sexes. In the females, the increase in absolute spleen weight was statistically significant at 10 and 80 mg/kg bw per day, whereas the increase in relative spleen weight was statistically significant at 25 and 80 mg/kg bw per day. Female adrenal weights were significantly greater than those of controls at 80 mg/kg bw per day on an absolute basis and at 25 and 80 mg/kg bw per day on a body weight–related basis. Dose-related histopathological changes were seen in the stomach. Squamous epithelial hyperplasia with hyperkeratosis, ulceration, chronic inflammation and oedema was observed in seven males and eight females in the 80 mg/kg bw per day group. Ulceration, chronic inflammation and oedema were seen in the stomachs of two males in the 25 mg/kg bw per day group. Extramedullary haematopoiesis was seen in two males and two females in the 80 mg/kg bw per day group, one male in the 25 mg/kg bw per day group and one female in the 10 mg/kg bw per day group; the authors concluded that only the cases in the high-dose group were likely to be treatment related. The authors concluded that the no-observed-adverse-effect level (NOAEL) was 10 mg sodium chlorite/kg bw per day, which is equal to 7.4 mg chlorite/kg bw per day (Harrington et al., 1995a).

Cats

Male mixed-breed cats (number not specified) received sodium chlorite in their drinking-water (length of time not specified) at concentrations up to 1000 mg/l, expressed as chlorite. Based on the assumptions of daily water consumption for cats of about 110 ml/day (Hawthorne & Markwell, 2004) and a body weight of 2 kg, the dose of sodium chlorite was estimated to be up to 55 mg/kg bw per day, although this could be affected by reduced consumption at higher concentrations due to the reduced palatability of the drinking-water containing the test substance. Significant decreases in haemoglobin and packed cell volumes were seen, but there was no increase in methaemoglobin. Red cell GSH levels were significantly reduced in all but the lowest dose group (10 mg/l). Within 2 weeks after cessation of treatment, packed cell volume returned to normal, and there was a trend towards recovery of haemoglobin concentrations. The authors concluded that the red cell was the most sensitive to damage by sodium chlorite and that erythrocytic GSH was the most susceptible parameter. Few other signs of chlorite toxicity were observed (Heffernan et al., 1979b).

Monkeys

Five adult male and seven adult female African Green monkeys were used to test the safety of solutions of chlorine dioxide (at concentrations of 0, 30, 100 and 200 mg/l), sodium chlorite and sodium chlorate (both at concentrations of 0, 25, 50, 100, 200 and 400 mg/l, expressed as ionic equivalents to chlorine dioxide) in drinking-water. If water consumption of 580 ml/day and a mean body weight of 5 kg are assumed, the sodium chlorite dose would be equivalent to 0, 3, 6, 12, 23 and 46 mg/kg bw per day, respectively; the authors cited the top dose as being equal to 58.4 ± 27.6 mg/kg bw per day. Each test substance was administered for 30–60 days at exponentially rising doses, followed by a 6- to 9-week resting period before the next substance was tested. Blood samples were taken at each dose changeover, then biweekly. Standard haematology tests were carried out for each blood sample. Dose-related statistically significant observations resulting from sodium chlorite administration were decreases in red cell count and cell indices, decreases in serum thyroxine (T_4) levels and haemoglobin levels, and increases in serum glutamate–pyruvate transaminase (SGPT) levels, reticulocytes and methaemoglobin. At the highest dose of chlorine dioxide (200 mg/l), erythema and ulceration of the oral mucosa, mucous nasal discharge and avoidance of drinking-water by the monkeys were noted. This high-dose study was terminated after 1 week because the monkeys showed signs of dehydration and azotaemia. A dose-dependent decrease in blood T_4 was seen in animals dosed with chlorine dioxide. At dose levels of about 9 mg/kg bw per day, the chlorine dioxide appeared to act as a potent inhibitor of thyroid synthesis. T_4 depression was statistically related to water consumption and chlorine dioxide dose. Since the animals' fluid intake was near normal (although values were not given), this effect was not considered to be due to dehydration. Sodium chlorite at most of the doses tested induced a self-compensating oxidative stress in the monkey haematopoiesis. About midway through exposure, a rebound effect occurred for haemoglobin and red cell synthesis. A dose-dependent rise in alanine aminotransferase (ALT) was detected in the monkeys, which may indicate accelerated hepatic activity during the transient oxidative haemolytic period. The authors concluded that thyroid inhibition is a significant health effect related to ingestion of chlorine dioxide (Bercz et al., 1982).

(c) Sodium chlorate

Rats

Sprague-Dawley rats (14 per sex per group) were administered sodium chlorate by gavage at doses of 0, 10, 100 or 1000 mg/kg bw per day for 3 months (equivalent to 0, 8, 79 and 788 mg chlorate/kg bw per day). No treatment-related effects were observed on mortality, physical appearance or behaviour, body weight, food consumption, clinical chemistry, gross necropsy or organ histopathology. At the highest dose, decreases in erythrocyte count, haemoglobin concentration and erythrocyte volume fraction (haematocrit) were observed, indicative of anaemia. A no-observed-effect level (NOEL) of 100 mg sodium chlorate/kg bw per day (79 mg/kg bw per day expressed as chlorate) was identified from this study (Bio/dynamics Inc., 1987b).

Sprague-Dawley rats (five per sex per group) were administered sodium chlorate in their drinking-water for 90 days at concentrations of 3, 12 or 48 mmol/l (249, 996 and 3984 mg/l). Based on measured water consumption, the doses of chlorate were equal to 30, 100 and 510 mg/kg bw per day for males and 42, 158 and 797 mg/kg bw per day for females. Body weight gain was decreased in both sexes in the high-dose group, and lower relative heart, kidney and liver weights were observed in the high-dose group in both sexes. Some decreases in haemoglobin, haematocrit and erythrocyte count were also observed in the high-dose group in both sexes. Pituitary lesions and thyroid gland colloid depletion were observed in both the mid- and high-dose groups of both sexes. A NOAEL of 30 mg/kg bw per day was identified from this study (McCauley et al., 1995).

Dogs

Beagle dogs (four per sex per dose) were administered sodium chlorate by gavage at doses of 0, 10, 60 or 360 mg/kg bw per day for 3 months (0, 8, 47 and 284 mg chlorate/kg bw per day). No significant effect was observed on body weight, food consumption, clinical chemistry, organ weights, ophthalmic examination, gross necropsy or tissue histopathology in all dose groups. A slight elevation in methaemoglobin level was observed at the high dose, but this was judged to be within normal limits and therefore not treatment related. A NOAEL of 360 mg/kg bw per day was identified (282 mg/kg bw per day expressed as chlorate) (Bio/dynamics Inc., 1987a).

Monkeys

Five adult male and seven adult female African Green monkeys were used to test the safety of solutions of sodium chlorate at concentrations of 0, 25, 50, 100, 200 and 400 mg/l, expressed as ionic equivalents to chlorine dioxide, in drinking-water. If water consumption of 580 ml/day and a mean body weight of 5 kg are assumed, the sodium chlorate consumption would be equivalent to 0, 3, 6, 12, 23 and 46 mg/kg bw per day, respectively; the authors cited the top dose as being equal to 54.2 ± 38 mg/kg bw per day. The test substance was administered for 30–60 days at exponentially rising doses, followed by a 6- to 9-week resting period before the next substance was tested. Blood samples were taken at each dose changeover, then biweekly. Standard haematology tests were carried out for each blood sample. Dose-related statistically significant observations resulting from sodium chlorate administration were decreases in erythrocyte count and cell indices, decreases in serum T_4 and haemoglobin, and increases in SGPT levels, reticulocytes and methaemoglobin. Sodium chlorate at most of the doses tested induced a self-compensating oxidative stress in the monkey haematopoiesis. About midway through exposure, a rebound effect occurred for haemoglobin and red cell synthesis, but this was not as marked as for sodium chlorite. A dose-dependent increase in ALT was detected in the monkeys, which may indicate accelerated hepatic activity during the transient oxidative haemolytic period (Bercz et al., 1982).

2.2.3 Long-term studies of toxicity and carcinogenicity

(a) Sodium chlorite

The International Agency for Research on Cancer (1991) concluded that sodium chlorite was not classifiable as to its carcinogenicity to humans (Group 3).

Mice

In a study of carcinogenicity and tumour-promoting effects, 0.2 ml sodium chlorite solution dissolved in acetone (20 mg/ml) was applied twice weekly to the shaven skin of 15–20 female Sencar mice for 51 weeks with and without prior initiation by topical application of 20 nmol dimethylbenzanthracene (DMBA). Further groups were treated with acetone for 51 weeks, with and without prior DMBA initiation. If a body weight of 20 g is assumed, the dose would be equivalent to 400 mg sodium chlorite/kg bw per week (43 mg/kg bw per day, expressed as chlorite). Six of the 20 mice receiving both DMBA and sodium chlorite developed skin tumours, whereas no tumours were observed in any of the other dose groups. This incidence was reported to be not statistically significant from control (Kurokawa et al., 1984).

Sodium chlorite (purity 99.4%) was administered in drinking-water at concentrations of 250 or 500 mg/l to groups of approximately 50 male and 50 female B6C3F1 mice for 85 weeks. If a body weight of 30 g per mouse and water consumption of 5 ml/day are assumed, doses of sodium chlorite would be equivalent to 42 and 83 mg/kg bw per day (31 and 62 mg/kg bw per day, expressed as chlorite), respectively. Animals were observed daily, and dead/moribund animals were autopsied immediately. At the end of the study, blood samples were taken from all surviving animals, and a necropsy was carried out. Body weight increases were comparable in all groups. Haematological changes were apparently not observed, but no data were presented. Statistical analysis of the tumour data was compromised by reduced survival in the control male group, resulting from severe fighting. Incidences of combined liver tumours were statistically significantly increased at the low dose but not the high dose (22/47 and 17/43, respectively, compared with 7/35 in controls). The incidence of hyperplastic nodules was significantly greater in both dose groups compared with controls, but greater at the low dose. In the high-dose males, there was a significant increase in the combined incidence of adenomas and adenocarcinomas (0/35, 3/47 and 7/43 at 0, 250 and 500 mg/l, respectively) and of adenomas of the lung (0/35, 2/47 and 5/43 at 0, 250 and 500 mg/l, respectively). No increases were reported in any tumour type in females. The authors noted that the observed incidences related to benign tumours and were within the historical control ranges, and they concluded that the results of this carcinogenicity test were inconclusive (Kurokawa et al., 1986).

Groups of 50 male and 50 female B6C3F1 mice were dosed with sodium chlorite (purity 82–87%) in the drinking-water at concentrations of 0, 250 or 500 mg/l for 80 weeks followed by distilled water alone for 5 weeks. Total sodium chlorite intakes over the 80 weeks calculated from mean water consumption data indicated average sodium chlorite doses of 33 and 53 mg/kg bw per day for females (25 and

39 mg/kg bw per day, expressed as chlorite) and 60 and 76 mg/kg bw per day for males (45 and 57 mg/kg bw per day, expressed as chlorite). Animals were observed daily and weighed weekly for 13 weeks and every 2 weeks after that. After 85 weeks, animals were sacrificed, gross findings recorded and the following organs weighed: brain, heart, lung, liver, pancreas, spleen, kidney, adrenals and testes or ovaries. These organs were examined histologically along with the salivary glands, trachea, thymus, lymph nodes, stomach, small intestine, large intestine, urinary bladder, pituitary, thyroid, prostate, seminal vesicle, uterus, vagina, mammary gland, skeletal muscle, eye, Harderian gland, spinal cord, bone and any tissue with abnormal appearance. Body weights, organ weights, water consumption and mortality did not show any treatment-related differences between groups. There were no significant differences in non-neoplastic lesions. The incidence of lung adenomas was significantly higher in the high-dose male group than in the control group (5/23 vs 0/35), and the incidence of malignant lymphoma/leukaemia was significantly decreased in the high-dose females (1/50 vs 7/47). No significant differences were found in the incidence of other tumours (Yokose et al., 1987).

Rats

Sodium chlorite (purity 99.4%) was administered at concentrations of 300 or 600 mg/l in drinking-water to groups of approximately 50 male and 50 female F344 rats for 85 weeks, when the study was prematurely terminated because of widespread infection with Sendai virus. Sodium chlorite intakes were reported to be 18 and 32 mg/kg bw per day for males and 28 and 41 mg/kg bw per day for females. At the end of the study, urine and blood samples were taken from all surviving animals and a necropsy was carried out. Pneumonia was found in all animals, and lung abscesses were found in some, but these were not thought to be treatment related. The urinalysis, haematological analysis and clinical chemistry tests showed no significant differences between treated and control animals except for serum glutamate–oxalate transaminase (SGOT), which was significantly reduced in high-dose males. No significant differences in incidence of tumour-bearing animals or rates of tumour development were observed between treated and control rats of either sex. The authors concluded that sodium chlorite was not carcinogenic in rats (Shimoyama et al., 1985; Kurokawa et al., 1986).

(b) Chlorate

Mice

Groups of 50 male and 50 female B6C3F1 mice were exposed to sodium chlorate at concentrations of 0, 0.5, 1.0 or 2.0 g/l in their drinking-water for 2 years. The average doses were equivalent to 40, 80 and 160 mg/kg bw per day for males and 30, 60 and 120 mg/kg bw per day for females. Survival rates and water consumption were similar between test and control groups throughout the study. Mean body weights of exposed females were generally less than the control group after week 84 of the study. In female mice, there was a positive trend in the incidence of pancreatic islet cell adenoma and carcinoma (combined), and thyroid gland follicular cell hypertrophy was significantly increased in high-dose females. The

incidences of bone marrow hyperplasia were significantly increased in all exposed females. The authors concluded that there was no evidence of carcinogenic activity of sodium chlorate in male mice at the doses tested and equivocal evidence of carcinogenic activity in female mice based on marginal increases in incidence of pancreatic islet neoplasms (National Toxicology Program, 2005).

Rats

Sodium and potassium chlorate were evaluated as promoters of renal tumours in groups of 15 male F344 rats. The initiation was by administration of *N*-ethyl-*N*-hydroxyethyl-nitrosamine at 500 mg/l in drinking-water 3 times a week for 2 weeks at the start of the study. Sodium chlorate was administered in the drinking-water at a concentration of 10 g/l for 25 weeks. Based on drinking-water consumption, the doses were reported to be 686 mg/kg bw per day in the initiated mice and 654 mg/kg bw per day in the mice consuming sodium chlorate without initiator (equivalent to 541 and 516 mg chlorate/kg bw per day). Two further groups consumed distilled water in place of the sodium chlorate with or without initiation. Chlorate administration resulted in no significant increases in incidence of dysplastic foci or renal cell tumours, compared with the relevant initiated or control groups (Kurokawa et al., 1985).

Groups of 50 male and 50 female F344/N rats were exposed to sodium chlorate in the drinking-water for 2 years at doses equivalent to approximately 5, 35 and 75 mg/kg bw per day for males and 5, 45 and 95 mg/kg bw per day for females. Survival rates, mean body weights and water consumption were similar between test and control groups throughout the study. There were positive trends in the incidence of thyroid gland follicular cell carcinoma in male rats and thyroid gland follicular cell adenoma and carcinoma (combined) in males and females. The incidence of thyroid gland follicular cell hypertrophy was significantly increased in all exposed male groups and in the two high-dose groups of females. Thyroid gland focal follicle mineralization occurred in most females in the mid- and high-dose groups. The incidences of haematopoietic cell proliferation in the spleen of high-dose males and bone marrow hyperplasia in the mid- and high-dose male groups were significantly greater than controls. The authors concluded that there was some evidence of carcinogenic activity of sodium chlorate in male and female rats based on increased incidence of thyroid gland neoplasms (National Toxicology Program, 2005).

Because a NOAEL was not identified in this study, the Committee decided to apply a benchmark dose (BMD) approach to derive a point of departure on the dose–response curve (see Annex 3 of reference *176* in Annex 1). A summary of the key neoplastic and non-neoplastic findings is shown in Table 4.

The United States Environmental Protection Agency (USEPA) BMD software version 1.4.1 (United States Environmental Protection Agency, 2007) was used for modelling the data on rat thyroid gland follicular cell hypertrophy, which was the effect observed at the lowest dose levels. A range of models were applied in order to derive the BMD for a 10% increase in follicular cell hypertrophy (BMD_{10}) and the corresponding 95% lower confidence limit ($BMDL_{10}$), as shown in

Table 4. Key neoplastic and non-neoplastic thyroid lesions in the 2-year carcinogenicity study of sodium chlorate in F344/N rats[a]

Sodium chlorate concentration in drinking-water (mg/l)	Approximate dose of sodium chlorate (mg/kg bw per day)	Survival	Thyroid gland follicular cell hypertrophy incidence	Follicular cell adenoma and carcinoma (combined) incidence
Males				
0	0	36/50	4/47	1/47
125	5	27/50	13/44	0/44
1000	35	31/50	33/43	0/42
2000	75	40/47	40/47	6/47
Females				
0	0	37/50	3/47	1/47
125	5	36/50	7/47	0/47
1000	45	33/50	27/43	1/43
2000	95	41/50	42/46	4/46

[a] From National Toxicology Program (2005).

Tables 5 and 6. The $BMDL_{10}$ values ranged from 1.1 to 4.4 mg chlorate/kg bw per day, with the lowest value representing the best fit.

2.2.4 Genotoxicity

A number of genotoxicity tests have been conducted using sodium chlorite and chlorate, but none with ASC (see Table 7). For sodium chlorite, in vitro studies include one positive and two negative reverse mutation assays and a positive chromosomal aberration assay. In vivo data include one positive and three negative micronucleus assays, a negative bone marrow chromosomal aberration assay and a negative assay for sperm head abnormalities. In view of the lack of detail in the publications, it is not possible to determine whether these studies were adequately conducted or to draw conclusions on the genotoxicity of sodium chlorite. Sodium chlorate has given positive results in bacterial mutation assays, but was negative in vivo.

2.2.5 Reproductive toxicity

(a) Acidified sodium chlorite: developmental toxicity

The germicidal gel product described in section 1.2 was applied topically to the shaven backs of groups of 20 pregnant Swiss Webster mice from gestation day (GD) 6 to GD 15 at 1 and 2 g/kg bw (395 and 790 mg sodium chlorite/kg bw, or 294

Table 5. BMD_{10} and $BMDL_{10}$ calculations for thyroid gland follicular cell hypertrophy in male F344/N rats administered sodium chlorate in drinking-water for 2 years

Model	Log (likelihood)	*p*-value	AIC	Chi-square	*P*-value	Accept	BMD_{10} (mg/kg bw per day)	$BMDL_{10}$ (mg/kg bw per day)
Full model	–83.49							
Gamma multistage	–85.46	0.14	174.9	4.13	0.13	??	3.5	2.8
Log-logistic	–83.61	0.65	173.2	0.24	0.65	Yes	1.9	1.1
Multistage	–85.46	0.14	174.9	4.13	0.13	??	3.5	2.8
Log-probit	–86.20	0.07	176.4	5.74	0.06	??	5.9	4.4
Quantal-linear	–85.46	0.14	174.9	4.13	0.13	??	3.5	2.8
Weibull	–85.46	0.13	174.9	4.13	0.13	??	3.5	2.8
Reduced model	–127.46							

AIC, Akaike Information Criterion.

and 588 mg chlorite/kg bw). Control groups either received placebo gel containing gelling agents but no active ingredients or were untreated. The mice were observed daily, weighed weekly and sacrificed on GD 18. Individual fetal weights, length and sex were recorded, and gross examination for external abnormalities was carried out for all live fetuses. Half of the fetuses from each litter were examined for soft tissue malformations, and the remainder for skeletal abnormalities. No statistically significant effects were reported in either the dams or the fetuses (Gerges et al., 1985).

In a similar study, the germicidal liquid was administered by gavage to groups of 20 Swiss Webster mice at doses equivalent to 132 and 198 mg sodium chlorite/kg bw per day (98 and 147 mg chlorite/kg bw). A negative control group received deionized water only, and a positive control group received 100 mg cortisone/kg bw. The mice were observed daily, weighed weekly and sacrificed on GD 18. Fetuses were removed, and the number of live and dead fetuses and the number of resorptions were recorded. The sex, weight and length of each fetus were recorded, and each was examined for gross external malformations. Half of the fetuses were examined for skeletal abnormalities, and the remainder for soft tissue abnormalities. No statistically significant effects were reported in either the dams or the fetuses (Skowronski et al., 1985).

The germicidal gel product was applied topically to the shaven backs of groups of 20 pregnant Sprague-Dawley rats from GD 6 to GD 15 at 1 and 2 g/kg

Table 6. BMD_{10} and $BMDL_{10}$ calculations for thyroid gland follicular cell hypertrophy in female F344/N rats administered sodium chlorate in drinking-water for 2 years

Model	Log (likelihood)	*p*-value	AIC	Chi-square	*P*-value	Accept	BMD_{10} (mg/kg bw per day)	$BMDL_{10}$ (mg/kg bw per day)
Full model	–72.19							
Gamma multistage	–73.03	0.62	152.1	0.24	0.62	Yes	6.4	3.7
Log-logistic	–73.67	0.21	153.4	1.54	0.21	Yes	12.6	3.0
Multistage	–73.92	0.89	151.8	0.02	0.89	Yes	6.0	3.8
Log-probit	–73.76	0.43	151.5	1.69	0.43	Yes	8.4	6.4
Quantal-linear	–73.17	0.77	150.3	0.50	0.78	Yes	4.7	3.7
Weibull	–72.99	0.69	152.0	0.16	0.69	Yes	6.5	3.8
Reduced model	–125.1	<0.001						

bw (395 and 790 mg sodium chlorite/kg bw, or 294 and 588 mg chlorite/kg bw). Control groups either received placebo gel containing gelling agents but no active ingredients or were untreated. Animals were observed daily and weighed weekly. On GD 20, the animals were sacrificed and the uterine contents examined. The numbers of live and dead fetuses and resorptions were recorded. Individual fetal weights, length and sex were recorded, and gross examination for external abnormalities was carried out. Half of the fetuses from each litter were examined for soft tissue malformations, and the remainder for skeletal abnormalities. No statistically significant effects were seen in either the dams or the fetuses (Gerges et al., 1985).

Similarly, the germicidal liquid product was administered by gavage to groups of 20 Sprague-Dawley rats at doses equivalent to 132 and 198 mg sodium chlorite/kg bw per day (98 and 147 mg chlorite/kg bw). A negative control group received deionized water only, and a positive control group received 250 mg sodium salicylate/kg bw. Animals were observed daily, weighed weekly and killed on GD 20. Fetuses were removed, and the numbers of live and dead fetuses and the number of resorptions were recorded. The sex, weight and length of each fetus were recorded, and each was examined for gross external malformations. Half of the fetuses were examined for skeletal abnormalities, and the remainder for soft tissue abnormalities. No statistically significant effects were seen in either the dams or the fetuses (Skowronski et al., 1985).

The germicidal gel product was applied topically to the shaven backs of groups of 12 pregnant New Zealand White rabbits on GD 6–18 of pregnancy as

Table 7. Results of assays for genotoxicity with sodium chlorite and related compounds

Test system	Test object	Concentration and test substance	Results	Reference
In vitro				
Reverse mutation[a]	*Salmonella typhimurium* TA92, TA94, TA98, TA100, TA1535, TA1537	0.3 mg sodium chlorite/plate (purity 88.8%)	Positive +S9 Negative –S9	Ishidate et al. (1984)
Reverse mutation[a]	*S. typhimurium* TA94, TA97, TA98, TA100, TA102, TA1537	0, 0.001, 0.005, 0.01, 0.05 and 0.1 mg sodium chlorite/ plate	Negative	Fujita & Sasaki (1987)
Reverse mutation[a]	*S. typhimurium* TA98, TA100	10, 20 and 30 mg chlorine dioxide/l added to fish samples[b]	Negative	Kim et al. (1999)
Reverse mutation[a]	*S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535	Sodium chlorate, concentration not specified	Negative ±S9	National Toxicology Program (2005)
Chromosomal aberration	Chinese hamster fibroblast cells	0.02 mg sodium chlorite/ml	Positive[c]	Ishidate et al. (1984)
Mutation	*Chlamydomonas reinhardtii*	100 mmol potassium chlorate/l	Positive	Prieto & Fernandez (1993)
Mutation	*Rhodobacter capsulatus*	100 mmol potassium chlorate/l	Positive	Prieto & Fernandez (1993)
Mutation	*S. typhimurium*	0, 5, 10, 50 and 100 mmol potassium chlorate/l	Negative	Prieto & Fernandez (1993)
In vivo				
Micronucleus formation	Mouse bone marrow	1 dose of 7.5, 15, 30 or 60 mg sodium chlorite/kg bw intraperitoneally	Positive[d]	Hayashi et al. (1988)
Micronucleus formation	Mouse bone marrow	4 doses in 24 h, 15 mg sodium chlorite/ kg bw intraperitoneally	Negative[e]	Hayashi et al. (1988)
Micronucleus formation	Mouse bone marrow	1 dose of 37.5, 75, 150 or 300 mg sodium chlorite/kg bw by gavage	Negative[d]	Hayashi et al. (1988)
Micronucleus formation	Mouse bone marrow	0.2, 0.5 and 1 mg of sodium chlorite/day for 5 days	Negative[f]	Meier et al. (1985)

Table 7 (contd)

Test system	Test object	Concentration and test substance	Results	Reference
		(equivalent to 10, 25 and 50 mg sodium chlorite/kg bw per day) by gavage		
Micronucleus formation	Mouse peripheral blood	Up to 350–365 mg sodium chlorate/kg bw per day in drinking-water for 3 weeks	Negative[f]	National Toxicology Program (2005)
Chromosomal aberration	Mouse bone marrow	0.2, 0.5 and 1 mg of sodium chlorite/day once or for 5 days (equivalent to 10, 25 and 50 mg sodium chlorite/kg bw per day) by gavage	Negative[g]	Meier et al. (1985)
Chromosomal aberration	Mouse bone marrow	0.2, 0.5 and 1 mg of sodium chlorate/day once or for 5 days (equivalent to 10, 25 and 50 mg sodium chlorate/kg bw per day) by gavage	Negative[g]	Meier et al. (1985)
Sperm head abnormalities	Mouse sperm	1 dose of 0.2, 0.5 or 1 mg of sodium chlorite (equivalent to 10, 25 and 50 mg sodium chlorite/kg bw per day) by gavage	Negative[h]	Meier et al. (1985)

S9, 9000 × *g* supernatant from rat liver.
[a] In the presence and absence of Kanechlor KC-400 induced rat liver microsomes.
[b] Chlorine dioxide added to seafood samples at ratio of 2:1. Chlorite was quantified in samples, and levels did not exceed the detection limit of 0.05 mg/l.
[c] 24- and 48-h sampling times.
[d] Killed at 18 h.
[e] Killed 24 h after final dose.
[f] Killed 6 h after final dose.
[g] Killed at 6 h after final dose or 6, 24 and 48 h after single dose.
[h] Killed 1, 3 and 5 weeks after final dose.

follows: high-dose gel, 2 g/kg bw (containing 4 times the concentration of sodium chlorite in the low-dose gel); low-dose gel, 2 g/kg; placebo gel (containing gelling

agents but no active ingredients), 2 g/kg bw; untreated controls. The concentration of sodium chlorite was not supplied. Rabbits were placed in semi-occlusive binders for 6 h following treatment, observed daily for signs of toxicity and weighed on GD 1, 6, 12, 18 and 29. All animals were sacrificed on day 29, and the uterine contents examined. All fetuses were sacrificed and examined for external visceral and skeletal abnormalities. Soft tissues were removed and examined in detail for anomalies, and fetuses were fixed and prepared for examination for skeletal defects. All pregnant rabbits were in good general health both during and after the treatment period. No changes were seen in the skin of the rabbits receiving the low-dose gel or placebo gel or the non-treated group. By GD 8, all animals treated with the high-dose gel had slight erythema; on GD 11, moderate to severe erythema was seen, which recovered by GD 18. The body weight gains in the low-dose and placebo gel groups were significantly decreased compared with controls. The ratio of total implants/corpora lutea was significantly increased in the high-dose gel group. The mean fetal weights and lengths of all gel groups were significantly reduced compared with controls, with no dose–response relationship. No treatment-related differences were seen in the fetal skeletons or soft tissues of test animals compared with controls. The authors suggested that the effects in this study may be attributable to the binders used on the rabbits following treatment (Abdel-Rahman et al., 1987c).

(b) Sodium chlorite: reproductive and developmental toxicity

(i) Multigeneration reproductive toxicity

A two-generation reproduction and developmental neurotoxicity study has been conducted in accordance with Good Laboratory Practice (GLP) using technical-grade sodium chlorite (purity 81.4%). For each generation, groups of 25–30 male and female Sprague-Dawley rats were administered sodium chlorite at analytically verified concentrations of 0, 35, 70 or 300 mg/l in their drinking-water for 10 weeks prior to mating, during mating and during gestation, or 0, 17.5, 35 or 150 mg/l during lactation to allow for the increased water consumption. The authors noted that these concentrations corresponded to sodium chlorite doses of approximately 0, 4, 8 and 30 mg/kg bw per day for male rats and 0, 5, 10 and 39 mg/kg bw per day for female rats (equal to 0, 3, 6 and 23 or 0, 3.8, 7.5 and 29 mg chlorite/kg bw per day, respectively). The rats were observed for clinical signs of toxicity, body weights, food and water consumption, estrous cyclicity, reproductive parameters, sperm motility and morphology. Necropsy was carried out on all animals, and microscopic evaluation of gross lesions and reproductive organs from animals in the high-dose and control groups and any animals with suspected reduced fertility was carried out. Body weight, viability, survival, landmarks of pup development and sexual maturation and gross external evaluations were assessed for all pups, and necropsy was performed for pups not selected for the F_1 generation. Additionally, F_1 and F_2 pups underwent a special necropsy on postnatal day (PND) 25, and the brain, liver, adrenals, spleen, thymus, testes and ovaries were weighed. Haematological evaluation took place on one male and one female pup from each F_1 litter soon after birth; at 13 weeks of age, all animals in the F_1 generation selected for breeding were assessed for haematology and serum thyroid hormones. The first

20 litters in each treatment group of the F_1 generation were selected for a functional observational battery of tests to assess normal behaviour, motor activity, swim maze and acoustic startle habituation. Ten animals from each sex per group in the F_1 generation underwent a neuropathological assessment.

No treatment-related clinical signs of toxicity were seen in the F_0 or F_1 generations. For F_0 parental animals, dose-related decreases in water consumption in the 70 and 300 mg/l groups were noted; in the F_1 generation, dose-related decreases were seen for males in all treatment groups and in females at 300 mg/l. Body weight and food consumption were significantly decreased for F_1 males in the 300 mg/l dose group. In high-dose females, body weights were reduced during the last week of gestation, at parturition and at some points during lactation compared with controls. A treatment-related reduction in pup body weights was observed for male and female F_1 and F_2 pups in the 300 mg/l dose group. Small but statistically significant increases in time to preputial separation in the F_1 pups of the 70 and 300 mg/l groups and in time to vaginal opening of the F_1 pups of the 300 mg/l group were observed. Neither observation was found in the F_2 pups. These changes were considered by the authors to be related to body weight. In the high-dose group, significant decreases in red blood cells, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were observed for all animals; in the 70 mg/l group, white blood cell count was significantly reduced. A statistically significant decrease in maximum startle response was seen in the 70 and 300 mg/l dose group pups on day 24 following birth, but the authors concluded that this was due to habituation of the control groups rather than any treatment-related effect. No reproductive toxicity or significant alterations in postnatal development or thyroid hormone levels were observed. The authors concluded that the lowest NOAEL for sodium chlorite was 70 mg/l in drinking-water or 8 mg/kg bw per day for males and 10 mg/kg bw per day for females (Gill et al., 2000). However, the World Health Organization (2004) considered that the effects on startle amplitude, decreased absolute brain weight in the F_1 and F_2 generations and altered liver weights in two generations were treatment related and concluded that the NOEL was 3 mg/kg bw per day expressed as chlorite.

(ii) Developmental toxicity

Groups of 10–12 female A/J mice were given 0 or 100 mg chlorite/l (in the form of sodium chlorite) in their drinking-water throughout pregnancy and lactation. Based on average drinking-water consumption, mean doses of chlorite were 0.62 and 1.53 mg/day during gestation and lactation, respectively. Pups were weighed at birth and weekly until weaning at 28 days. The conception rate for the treatment group was 39% and for the controls was 56%. There was a statistically significant decrease in average weight of pups at weaning (10.7 g in the treatment group and 12.5 g in the control group) and the average birth to weaning growth rate (0.336 g/day in the treatment group and 0.408 g/day in the control group). There were no statistically significant differences between the control and treatment groups (Moore & Calabrese, 1982).

Sodium chlorite was administered to pregnant female Sprague-Dawley rats via intraperitoneal injection, gavage or drinking-water. The intraperitoneal injection group of animals received 10 mg/kg bw per day on GD 8–15 (13 rats), 20 mg/kg bw per day on GD 8–15 (10 rats) or 50 mg/kg bw per day on GD 8–10 or 16–17 (7 rats). The animals dosed by gavage received 200 mg/kg bw per day on GD 8–10. Concentrations in drinking-water were 0.1%, 0.5% or 2% (equal to sodium chlorite doses of 106, 500 and 564 mg/kg bw per day, respectively, or 79, 372 and 420 mg/kg bw per day expressed as chlorite) on GD 8–15 (10 animals per group). Some rats underwent caesarean section on day 22, and others were allowed to deliver at term. Litter size, number of living and dead pups, weights of pups and crown–rump measurements were recorded. Offspring were examined for gross malformations and then sacrificed. Half underwent examination of soft tissues, and the remainder underwent examination for skeletal malformations. Six pups from each litter were selected at random, and their postnatal growth was recorded at days 1, 8, 15, 22 and 29.

Intraperitoneal injection of sodium chlorite resulted in deaths, vaginal and urethral bleeding and weight loss at 20 and 50 mg/kg bw per day. At 10 mg/kg bw per day intraperitoneal injection, there were no deaths or bleeding, but weight loss was still observed. Effects of gavage at 200 mg/kg bw per day were similar to those seen at 50 mg/kg bw per day intraperitoneal injection. Following administration in drinking-water, body weight gain of the dams was decreased at 0.5% and 2% sodium chlorite, but increased at 0.1%, compared with controls. Water consumption was significantly reduced during the treatment period in all groups compared with controls, and food consumption was reduced in all except the 0.1% sodium chlorite group. Irregular blood cells, ruptured cells and haemolysis were observed in the dams receiving 2% sodium chlorite. In the offspring, the crown–rump distance was significantly decreased in all treatment groups. Increased numbers of dead and resorbed fetuses were reported, particularly at the higher doses, but there were no differences in postnatal growth of pups or the numbers of skeletal or soft tissue malformations (Couri et al., 1982).

Groups of 12 male Long-Evans rats were administered sodium chlorite at 0, 1, 10 or 100 mg/l in their drinking-water for 56 days prior to breeding and during the 10-day breeding period. Female rats (24 per dose group) were given the same concentrations for 14 days prior to breeding and throughout breeding, gestation and lactation until the pups were weaned on day 21. Water consumption was recorded but not reported. Following the breeding period, blood samples were taken and a gross necropsy was carried out on the male rats (organs not specified). The testis, epididymis, prostate and seminal vesicles were removed, weighed and examined histopathologically, except for the right cauda epididymis, which was minced, following which the sperm cells were examined for motility and abnormalities and counted. Dams were observed for fertility, length of gestation, body weight gain and maternal behaviour. At sacrifice, 21 days after birth, blood samples were taken from the dams, and a gross necropsy was carried out (organs not specified). The reproductive tract was removed for histopathological examination. Litters were evaluated for viability, litter size (~13 per dam), day of eye opening, body weight gain and gross external abnormalities. At necropsy, 10 pups per sex per dose were

bled for complete blood counts and hormone analysis, and the reproductive tracts were weighed and preserved. Selected pups were retained for observation of the day of vaginal patency and further hormone analysis. Male and female pups, but not the parental generation, in the 100 mg sodium chlorite/l group showed consistent reductions in triiodothyronine (T_3) and T_4 levels. There were no other statistically significant effects.

In a second study, four groups of 12 male rats consumed sodium chlorite at concentrations of 0, 100 or 500 mg/l in their drinking-water for 72–76 days. At necropsy, blood samples were taken and methaemoglobin and hormone analyses were carried out. Sperm count, motility, direct progressive movement and sperm head abnormalities were assessed. Water consumption was significantly reduced in the 500 mg/l group, although body weights were unaffected. No other treatment-related effects were reported.

A third study was carried out using three groups of male rats consuming 0, 10 or 100 mg sodium chlorite/l in their drinking-water. Observed fertility rates did not differ in a dose-dependent manner, and litter survival rates, mean litter size, median day of eye opening and observed vaginal patency were unaffected by the treatment. No compound-related effects were seen during necropsy or on histopathological examination or in the haematological parameters tested. Sperm count and per cent sperm motility were unaltered by sodium chlorite exposure.

When assessing the three studies together, in the 100 and 500 mg/l dose groups, a trend towards decreased direct progressive movement of sperm cells was noted, which, while not significant in individual experiments, was found to be significant when the results of the three experiments were combined. A significant increase in abnormal sperm forms was observed in the 100 and 500 mg/l dose group rats. At PND 21 and onwards in the pups, T_3 and T_4 levels were significantly reduced in animals exposed to 100 mg sodium chlorite/l or more (Carlton et al., 1987; George et al., 1987).

If water consumption of 25 ml/day and a mean body weight of 500 g are assumed, the sodium chlorite consumption would be 0.05, 0.5, 5 and 25 mg/kg bw per day for the 1, 10, 100 and 500 mg/l dose groups, respectively (0.037, 0.37, 3.7 and 18.5 mg/kg bw per day expressed as chlorite). These three studies indicate a NOEL of 0.5 mg/kg bw per day, or 0.37 mg/kg bw per day expressed as chlorite, and a lowest-observed-effect level (LOEL) of 5 mg/kg bw per day, or 3.7 mg/kg bw per day expressed as chlorite, although the accuracy of these doses is dependent on the assumptions made.

In a study reported in abstract form only, pregnant Wistar rats were dosed by gavage with 0, 25, 50 or 100 mg sodium chlorite/kg bw per day from GD 6 to GD 15. Rats were sacrificed on day 20 of pregnancy, and fetuses were examined. Decreased food consumption, anaemia, sedation, haematuria and death were seen in the high-dose rats, but no fetal effects were observed at any dose. The authors concluded that sodium chlorite was not teratogenic when administered orally and that the NOELs were 50 mg/kg bw per day for maternal toxicity and 100 mg/kg bw per day for the fetuses (Sakemi et al., 1999).

Groups of 16 pregnant New Zealand White rabbits received 0, 200, 600 or 1200 mg sodium chlorite/l (corrected for the purity of 80.6%) in their drinking-water from GD 7 to GD 19. Based on water consumption, which decreased with increasing sodium chlorite concentration, the calculated average intakes were reported to be equal to 0, 13, 35.5 and 53.1 mg sodium chlorite/kg bw per day, or 0, 9.8, 26.6 and 39.8 mg chlorite/kg bw per day, respectively. Clinical condition, body weight and food and water consumption were recorded until necropsy on GD 28. Live fetuses were weighed and examined for abnormalities. During GD 7–11, there was a significant dose-related decrease in food consumption in the 600 mg/l and 1200 mg/l groups and an associated decrease in body weight in the 1200 mg/l group, but no statistically significant changes in absolute body weights throughout the study. No treatment-related abnormalities were observed at maternal necropsy, and the mean number of corpora lutea, implantations, preimplantation losses and live fetuses and sex distribution were similar in all groups. The 600 and 1200 mg/l groups showed an increase in minor skeletal abnormalities and skeletal variants related to incomplete ossification of the pubis and sternebrae. No other treatment-related abnormalities were observed. The authors concluded that there was evidence of a slight effect of sodium chlorite on embryonic growth retardation and development, which was considered to be due to the maternal effects of sodium chlorite rather than direct action on the fetus (Harrington et al., 1995b). This study indicates a NOEL of 13 mg sodium chlorite/kg bw per day, or 9.8 mg chlorite/kg bw per day.

(c) Sodium chlorate: developmental toxicity

Sodium chlorate was administered to pregnant CD rats by gavage at doses of 0, 10, 100 or 1000 mg/kg bw per day on days 6–15 of gestation. No treatment-related effects were observed in maternal body weight gain, food consumption, clinical observation, number of implantations or gross necropsy. Examinations of fetuses on day 20 showed no effects on fetal weight or sex ratio, and no external, visceral or skeletal abnormalities were detected. A NOAEL of 1000 mg/kg bw per day was identified from this study (Bio/dynamics Inc., 1987b).

2.2.6 Special studies

(a) Blood

Individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency have been identified as being especially sensitive to the effects of oxidant stress such as that caused by chlorine dioxide and chlorite, because they have a reduced ability to form GSH. Two strains of mice, C57L/J (low G6PD activity) and A/J (high G6PD activity), have been used to study the effects of chlorite. Sodium chlorite was administered orally by gavage to groups of at least 22 mice (sex not specified) at 0 or 200 mg/kg bw at 12-h intervals. Animals were killed after the third dose, and the blood was analysed for haematological parameters, osmotic fragility and G6PD in the erythrocytes. Some groups of animals had 2 cm of tail severed after the second dose, in order to examine clot formation. Significant differences were seen between the two strains of mice receiving no treatment, with red blood cells, haematocrit and reticulocyte count being significantly higher in the C57L/J strain than in the A/J

strain, whereas G6PD activity was significantly lower in the C57L/J strain. Sodium chlorite treatment resulted in significantly decreased erythrocytes and haematocrit in C57L/J mice, but not in A/J mice. Reticulocyte counts were significantly increased in both strains. The difference between control and tests was most marked in the animals with cut tails. The authors concluded that the use of chlorite may induce haemolytic episodes or anaemia in G6PD-deficient individuals, as is shown in this G6PD-deficient mouse model (Moore & Calabrese, 1979).

C57L/J and A/J mice (10 or 15 per group, sex not specified) were given 100 mg chlorine dioxide/l or 0, 1, 10 or 100 mg sodium chlorite/l in their drinking-water for 30 days. Water consumption and body weights were recorded. At the end of the treatment period, the animals were sacrificed, and their blood was collected for measurement of the blood parameters G6PD activity, osmotic fragility and reduced GSH level. Electron microscopy was also carried out on whole blood samples. There were no differences in water consumption or body weight gain between the groups. No treatment-related effects were observed with chlorine dioxide treatment. The mice from both strains receiving sodium chlorite at 100 mg/l showed an increase in G6PD activity, mean corpuscular volume and osmotic fragility. The electron microscopy showed an increase in acanthocyte numbers in both strains of mice receiving 100 mg sodium chlorite/l. No strain versus treatment interactions were found, indicating that both strains reacted in the same way to the treatment. The authors concluded that sodium chlorite caused damage to the erythrocyte membranes, but not to the internal cell mechanisms (Moore & Calabrese, 1980).

AJ and C57L/J mice (10 per group, sex not specified) were administered sodium chlorite in their drinking-water for 30 days at levels of 0, 1, 10 or 100 mg/l. Following the treatment period, the animals were sacrificed, and their blood was tested for haematological parameters. G6PD activity, mean corpuscular volume and osmotic fragility were increased in both strains receiving 100 mg sodium chlorite/l. No significant effects were seen in the other dose groups. A significant difference between strains was noted in G6PD activity and osmotic fragility, but no significant strain versus treatment effects were noted. No differences were noted in water or food consumption between the groups (Moore & Calabrese, 1982).

If it is assumed that water consumption was 5 ml per animal per day and mean body weight was 30 g, the sodium chlorite intakes would be equivalent to 0.17, 1.7 and 17 mg/kg bw per day for the 1, 10 and 100 mg/l dose groups (0.31, 1.3 and 13 mg/kg bw per day, expressed as chlorite).

(b) Thyroid: sodium chlorate

Mice

Groups of 10 male and 10 female B6C3F1 mice were exposed to sodium chlorate at concentrations of 0, 0.125, 0.25, 0.50, 1.0 or 2.0 g/l in the drinking-water for 21 days. The average doses were equivalent to 0, 20, 45, 90, 175 and 350 mg/kg bw per day for males and 0, 20, 45, 95, 190 and 365 mg/kg bw per day for females. All mice survived the duration of the study, and mean body weights and water consumption were similar between test and control groups. No treatment-

related effects were observed in the thyroid or other tissues (National Toxicology Program, 2005).

Female B6C3F1 mice (six per group) were administered sodium chlorate at concentrations of 0, 0.5, 1.0, 2.0, 4.0 and 6.0 g/l in the drinking-water for 105 days, equivalent to about 0, 50, 100, 200, 400 and 600 mg/kg bw per day expressed as chlorate. There were no effects on thyroid histology, but no other details were provided (Hooth et al., 2001).

Rats

Groups of 10 male and 10 female F344/N rats were exposed to sodium chlorate at concentrations of 0, 0.125, 0.25, 0.50, 1.0 or 2.0 g/l in the drinking-water for 21 days. The average doses were equivalent to 0, 20, 35, 75, 170 and 300 mg/kg bw per day for males and 0, 20, 40, 75, 150 and 340 mg/kg bw per day for females (0, 16, 27, 59, 133 and 234 mg chlorate/kg bw per day and 0, 16, 31, 59, 117 and 265 mg chlorate/kg bw per day, respectively). All rats survived the duration of the study, and mean body weights and water consumption were similar between test and control groups. A dose-related decrease in segmented neutrophil counts occurred in rats of both sexes on days 4 and 22, and heart weights were significantly decreased in high-dose males. The incidence of minimal to mild thyroid gland follicular hypertrophy was significantly increased in rats exposed to doses of 75 mg/kg bw per day or greater (Hooth et al., 2001).

Groups of female F344 rats were exposed to sodium chlorate at concentrations of 0, 0.125, 1.0 or 2.0 g/l in the drinking-water for 90 days (n = 10) or to 0, 0.5, 1.0, 2.0, 4.0 and 6.0 g/l for 105 days (n = 6), equivalent to about 0, 5, 40 and 80 or 0, 20, 40, 80, 150 and 230 mg chlorate/kg bw per day, respectively. Serum thyroid stimulating hormone (TSH) was elevated at the highest dose, but T_3 and T_4 were not altered by treatment. There was an increased incidence of thyroid colloid depletion and follicular cell hyperplasia at doses of 2 g/l and above. Male F344 rats (n = 10) were administered sodium chlorate at 0, 0.001, 0.01, 0.1, 0.125, 1.0 or 2.0 g/l in the drinking-water for 90 days, equivalent to about 0, 0.05, 0.5, 5, 6, 50 and 100 mg/kg bw per day. Thyroid hypertrophy and decreased colloid were observed in animals of all treatment groups. In addition, there were dose-related incidence and severity of follicular cell hyperplasia at the two highest doses. Serum TSH was elevated at the highest dose, but T_3 and T_4 were not altered by treatment (Hooth et al., 2001).

(c) Nephrotoxicity

The effects of sodium chlorite on renal structure were investigated using sodium chlorite in drinking-water. Groups of mice received sodium chlorite at 0, 4, 20 or 100 mg/l for 30, 60 or 90 days. A high-salt control group received 100 mg sodium chloride/l. Following treatment, the kidneys were removed for examination by light microscopy and transmission electron microscopy. There were no statistically significant treatment-related differences in body weight gain, absolute or relative kidney weight or water consumption. Statistically significant time differences were observed in body weight change, absolute kidney weight and

weekly water consumption, but not in relative kidney weight. No significant interaction was found between length of treatment exposures and dose levels. No abnormalities were observed in the kidneys from any of the animals (Moore & Calabrese, 1982).

If it is assumed that water consumption was 5 ml/day and mean body weight was 30 g, the sodium chlorite intakes would be equivalent to 0.7, 3.3 and 16.7 mg/kg bw per day for the 4, 20 and 100 mg/l dose groups (0.5, 2.5 and 12.4 mg/kg bw per day, expressed as chlorite).

Groups of male Sprague-Dawley rats were administered sodium chlorite for 30, 90 or 180 days at 0, 31.2, 125 or 500 mg/l (five rats per group) or sodium chloride at 500 mg/l (10 rats per group) in their drinking-water. Positive control animals received a single intraperitoneal dose of mercury(II) chloride (1.5 mg/kg bw), which produces renal tubular necrosis in the rat. Following the exposure period, animals were sacrificed, the kidneys were prepared for histological examination and urinalysis was carried out. No significant differences in body weight changes or water consumption were seen between treatment groups. A significant increase in mean absolute and relative kidney weights was associated with the higher concentrations of sodium chlorite and sodium chloride at 30 and 90 days, but not at 180 days. No differences were seen between the high sodium chlorite and high sodium chloride groups. The kidneys of the treatment groups appeared normal following histopathological analysis. Positive control kidneys showed necrosis as expected and abnormal urinalysis results. No treatment-related differences were seen in the urinalysis of treatment and control animals. The authors concluded that sodium chlorite did not cause nephrotoxicity in this study (Moore et al., 1984).

If it is assumed that water consumption was 25 ml/day and mean body weight was 500 g, the sodium chlorite intakes would be equivalent to 1.6, 6.3 and 25 mg/kg bw per day for the 31.2, 125 and 500 mg/l dose groups (1.2, 4.7 and 18 mg/kg bw per day, expressed as chlorite).

Groups of 11–12 male C57L/J mice were administered 0, 4, 20 or 100 mg sodium chlorite/l and 100 mg sodium chloride/l as a high-salt control in their drinking-water for 30, 90 and 180 days. The sodium chlorite doses were equal to 1.0, 4.7 and 23 mg/kg bw per day at 30 days, 0.9, 4.2 and 22 mg/kg bw per day at 90 days and 0.9, 4.9 and 23 mg/kg bw per day at 180 days (approximately 0.7, 3.5 and 16 mg/kg bw per day, expressed as chlorite, at all time points). Upon completion of the treatment period, animals were weighed and sacrificed, and the kidneys were removed and examined using light and transmission electron microscopy. No treatment-related effects were observed, and the kidneys appeared normal (Connor et al., 1985).

(d) Sperm quality

Male B6C3F1 mice (10 per group) were administered total doses of 0.2, 0.5 or 1 mg sodium chlorite by gavage in five equal daily doses. Positive controls received 200 mg ethyl methanesulfonate/kg bw intraperitoneally in five equal doses. Animals were sacrificed 1, 3 and 5 weeks after dosing, and the caudae epididymides were removed and diced in saline. Tissue fragments were filtered out, and 1000

sperm heads were scored per animal for abnormalities. No treatment-related effects were observed (Meier et al., 1985).

Male Sprague-Dawley rats (six per group) were given a single dose of 200 mg sodium chlorite/kg bw by gavage and sacrificed after 2 or 14 days or five daily doses of 100 mg/kg bw on days 1–5 and sacrificed on day 8 or 17. The testis, epididymis, seminal vesicles and prostate were excised and weighed, and sperm number, motility and morphology were assessed. Sodium chlorite was found to have no effect on organ weights or sperm quality (Linder et al., 1992).

(e) Immune system

A number of immune activity assays were conducted in female B6C3F1 mice. Groups of eight mice received sodium chlorite in their drinking-water at concentrations of 0, 0.1, 1, 5, 15 or 30 mg/l for 28 days. Based on the average body weights and water consumption data provided, these concentrations were equal to doses of approximately 0, 0.02, 0.2, 0.8, 2.5 and 5 mg sodium chlorite/kg bw per day, or 0, 0.015, 0.15, 0.6, 1.9 and 3.8 mg/kg bw per day expressed as chlorite. At termination of the treatment, animals were assessed for immunomodulation using a number of assays, blood parameters and organ weights. There were no treatment-related differences in weight gain or water consumption and no overt signs of toxicity. Significant increases in absolute weights of liver, spleen and kidney were seen at all concentrations of sodium chlorite, but relative organ weights did not differ from control except for the spleen in the 5 mg/l group. Haematological parameters were generally unaffected by treatment. A significant increase in reticulocytes was seen in the 15 mg/l group, but not in other groups. The number of $CD8^+$ T cells was significantly increased in the highest treatment group. The total number of $CD4^+$ T cells, B cells, natural killer (NK) cells and macrophages and the total number of spleen cells remained unaffected by treatment. Spleen immunoglobulin M (IgM) antibody response to T-dependent antigen from sheep erythrocytes was determined using a modified haemolytic plaque assay, and antibody forming cells (AFC) were counted and expressed as AFC/10^6 spleen cells or AFC/spleen. Serum IgM antibody levels were assessed in the same animals by enzyme-linked immunosorbent assay (ELISA). Sodium chlorite did not significantly affect the primary AFC response to sheep erythrocytes in the individual treatment groups; however, an increasing trend in the AFC response was observed over the range of doses when the data were expressed as specific activity (AFC/10^6 spleen cells and AFC/spleen). Spleen cell numbers and serum IgM following exposure to sheep erythrocytes were unaffected by the treatment; therefore, the authors concluded that the increasing trend in the AFC response was not a significant change. Spleen cell mixed leukocyte response to stimulator DBA/2 spleen cells was assessed and expressed as counts per minute (cpm)/1 $\times$ 10^5 cells. No change in spleen cell response was observed following treatment with sodium chlorite. Peritoneal macrophage activation was assessed by intraperitoneal injection of 1 ml 10% thioglycollate to recruit macrophages into the peritoneal cavity. Peritoneal cells were collected following sacrifice and cultured with stimulants and then B16F10 tumour cells. Tumour cell proliferation was measured using a liquid scintillation counter and measured as the per cent suppression of tumour cell proliferation. Sodium chlorite

did not affect the ability of unstimulated and stimulated macrophages to suppress tumour cell proliferation. Basal and augmented NK cell activities in the spleen were measured following administration of 0.03 mg polyinosinic-polycytidylic acid to the mice 24 h prior to sacrifice to augment the NK cell activity. Radiolabelled YAC-1 target cells were used to induce activity in the NK cells, and the amount of radioactivity was measured. Results were presented as per cent cytotoxicity, and this was used to calculate the lytic units (number of splenocytes required to kill 25% of the target cells). No change in basal NK cell activity was observed at any effector to target cell ratio following treatment with sodium chlorite. Polyinosinic-polycytidylic acid augmented response was significantly suppressed at 0.1 mg sodium chlorite/l, but no effect was observed in other dose groups. The authors concluded that sodium chlorite produced minimal immunotoxic effects in mice (Karrow et al., 2001).

Male BDIX rats (20 per group) were dosed by intraperitoneal injection at 1 ml/kg bw per day of the following: saline solution for 12 days, WF10 (containing tetrachlorodecaoxygen as an active ingredient with 4.25% chlorite) for 12 or 90 days or sodium chlorite (62 mmol/l, equal to 5.6 mg sodium chlorite/kg bw per day, or 4.2 mg/kg bw per day expressed as chlorite) for 12 days or 90 days. Body weights and peripheral blood counts and haemoglobin were monitored during dosing, and the animals were sacrificed 24 h after the final dose. Following necropsy, cytochemical cell kinetics in bone marrow (5-bromo-2′-deoxyuridine) and histopathology of the spleen and bone marrow were examined. Mild anaemia was observed with both WF10 and sodium chlorite, with the lowest values seen on days 6 and 9. In rats treated with sodium chlorite, the anaemia persisted until the end of the study, whereas the animals receiving WF10 recovered. An increase in leukocyte count was seen in all test groups. An increase in monocytes was seen in the groups receiving WF10, but a decrease was seen in the groups receiving sodium chlorite as the dosing period increased. Examination of the bone marrow showed a consistent increase in DNA synthesizing bone marrow cells with WF10, although this returned to normal towards the end of the dosing period. WF10 treatment resulted in an increase in the proportion of mature granulocytes in the bone marrow, whereas sodium chlorite led to a significant decrease. Enlargement of the spleen was found following necropsy of all test animals dosed for 12 days, and histological analysis showed subcapsular focal proliferation of large mononuclear cells in the spleens of these animals. Long-term dosing with sodium chlorite produced severe splenomegaly, which was not seen following long-term dosing with WF10. Two animals from group 5 died during the study and showed signs of toxic pulmonary oedema, hydrothorax and fibrin deposits on the pleura and pericardium (Kempf et al., 1993).

2.3 Observations in humans

2.3.1 Case reports

A 49-year-old woman presented at a clinic with a dry irritating cough, dyspnoea and tachypnoea together with crepitation in the left apical region and gurgling or bubbling rales following inhalation of a bleaching solution containing sodium chlorite, formic acid and boiling water. Tachycardia and leukocytosis were

also recorded. A chest X-ray showed normal cardiovascular findings, but pulmonary function tests showed marked restrictive and obstructive ventilatory impairment with hypoxaemia. Corticosteroid therapy produced an improvement in the cough and dyspnoea, but treatment for obstruction produced no improvement in pulmonary function after 5 days. Recovery was slow, and all functional parameters returned to normal within 2 months, with occasional coughing and dyspnoea reported by the patient (Exner-Freisfeld et al., 1988).

A 25-year-old Chinese male was admitted to hospital with generalized cyanosis and respiratory distress following ingestion of 10 g sodium chlorite (equivalent to 167 mg/kg bw in a 60-kg adult). Methaemoglobinaemia was found, and intravenous methylene blue was given repeatedly; however, an acute haemolytic crisis occurred. Following this, the patient was put on constant haemodialysis to correct the fluid overload and remove metabolites of sodium chlorite. After 24 h, the methaemoglobin was reduced significantly, but the patient experienced acute renal failure. Haemodialysis continued for 4 weeks; after 3 months, renal function returned to normal (Lin & Lim, 1993).

The use of sodium chlorate as a weed killer has meant that a number of case reports concerning ingestion of large amounts of sodium chlorate are available. A review of 14 cases of sodium chlorate poisoning in patients whose ages ranged from 3 to 55 years demonstrated that doses in excess of 100 g (or 79 g as chlorate) were uniformly fatal. The lowest fatal dose was 15 g, equivalent to 196 mg chlorate/kg bw in a 60-kg person (Helliwell & Nunn, 1979).

2.3.2 Volunteer studies

A series of studies have been conducted in human volunteers in a single laboratory. Written informed consent was obtained from each subject prior to initiation of treatment.

Normal healthy adult male volunteers (10 per group) were given water containing no disinfectant or water containing chlorine dioxide, chlorite, chlorate, chlorine or chloramine at increasing concentrations over a series of six dosing periods of 3 days each. On the first day of each 3-day period, each volunteer ingested 1000 ml of dilute sodium chlorite solution in two portions taken 4 h apart. The chlorite concentrations were 0.01 mg/l on day 1, 0.1 mg/l on day 4, 0.5 mg/l on day 7, 1.0 mg/l on day 10, 1.8 mg/l on day 13 and 2.4 mg/l on day 16. If one assumes a mean body weight of 70 kg, the doses were equivalent to 0.000 14, 0.0014, 0.0071, 0.014, 0.026 and 0.034 mg/kg bw, respectively, expressed as chlorite. Individuals underwent an examination, including blood and urine analysis, electrocardiograms and physical examination (e.g. blood pressure, respiration rate, pulse and temperature). No treatment-related effects were noted (Lubbers et al., 1981, 1982; Lubbers & Bianchine, 1984).

Normal healthy adult male volunteers (10 per group) were then given 500 ml of water containing chlorine dioxide, chlorite, chlorate, chlorine or chloramine at 0 or 5 mg/l daily for 12 weeks. If one assumes a mean body weight of 70 kg, the dose of chlorite was equivalent to 0.036 mg/kg bw per day. Examinations included serum chemistry, blood count, urinalysis, special tests (e.g. thyroid hormones,

methaemoglobin, G6PD and electrocardiograms) and physical examination (e.g. blood pressure, respiration rate, pulse and temperature). The clinical evaluation did not show any treatment-related effects except for a statistically significant treatment group interaction in the mean corpuscular haemoglobin values for chlorite and chlorate. The minor subjective symptoms reported by the volunteers were not considered to be treatment related (Lubbers et al., 1981, 1982, 1984a).

Individuals with G6PD deficiency have been identified as likely to be especially sensitive to the effects of oxidant stress such as that caused by chlorine dioxide and chlorite, because they have a reduced ability to form GSH. Three healthy adult males with G6PD deficiency were given 500 ml of sodium chlorite at a concentration of 5 mg chlorite/l daily for 12 weeks. If one assumes a mean body weight of 70 kg, the dose of chlorite was equivalent to 0.036 mg/kg bw per day. Examinations during the treatment period and for the following 8 weeks included serum chemistry, blood count, urinalysis, special tests (e.g. methaemoglobin, G6PD and electrocardiograms) and physical examination (e.g. blood pressure, respiration rate, pulse and temperature). A mild suppression of mean corpuscular haemoglobin concentrations was observed in all individuals during weeks 2–4; the values then returned to the normal range for the remainder of the study period. The small number of individuals in this group meant that statistical analysis of the results was not possible (Lubbers et al., 1981, 1982, 1984b).

2.3.3 Population studies

In the United States of America (USA), chlorine dioxide was used in several states in the 1940s. Records from this time were used to study the effects of chlorine dioxide in drinking-water on infant health over a 1-year period when chlorine dioxide use was at its highest. Two geographical areas located close to each other and sharing some of the same maternity facilities were chosen. The highest levels of sodium chlorite were added to the Chicopee (Massachusetts) water supply in 1945, with a monthly average of 0.32 mg/l. Holyoke, an area in Massachusetts where chlorine dioxide was not used to treat drinking-water and whose residents had a similar number and demographic, was chosen as the control. Parameters such as location of birth (identifying hospital or at home), prematurity, birth weight, type of food given, mean age of mother and mean vitamin K dose were recorded for each case. A greater proportion of infants from Chicopee were judged to be premature. Although this differed by hospital of birth, the finding was independent of other factors and considered significant. The rates of jaundice, birth defects and fetal and neonatal mortality did not differ significantly between the communities (Tuthill et al., 1982).

Blood donor records were used to select 124 male volunteers in Sardinia comprising 60 with the normal G6PD phenotype and 64 with the G6PD-deficient phenotype. Individuals with abnormal blood chemistry parameters and possible cases of thalassaemia were excluded from the study. Individuals were interviewed, and personal details, place of residence and type of drinking-water normally consumed were recorded. Blood samples were taken, and the following blood parameters were measured: red blood cells, mean corpuscular haemoglobin, mean corpuscular volume, white blood cells, indirect bilirubinaemia and serum ferritin. The

results of a wide-ranging survey measuring the chlorite levels from water samples from across the water distribution networks were used to assess the potential chlorite intakes of the volunteers who drank tap water. A two-way analysis of variance (ANOVA) was carried out to identify the interactions between the type of water consumed and the G6PD phenotype, and regression was used to compare the blood chemistry results with the chlorite content in the drinking-water to get an indication of the dose–response relationship. Individuals with a normal G6PD phenotype who consumed tap water were found to have a slight but significant change in mean corpuscular volume and mean corpuscular haemoglobin when compared with individuals with a normal G6PD phenotype who consumed bottled water not treated with chlorites. The authors concluded that this is probably caused by oxidative stress due to chlorite ingestion. Individuals with a G6PD-deficient phenotype, who in general showed a reduction in red blood cells, haemoglobin and haematocrit and an increase in mean corpuscular volume and indirect bilirubin, showed no further changes attributable to the type of water they consumed. The authors therefore considered that oxidative stress caused by G6PD deficiency was not exacerbated by the consumption of water treated with chlorites. The authors acknowledged that the statistical power of the study was not sufficient to identify a dose–response relationship (Contu et al., 2005).

A case–control study with incident cases was performed in nine Italian towns between 1999 and September 2000. In total, 1194 subjects were studied, of which 343 had preterm births (26th–37th week), 239 had infants who were small for gestational age and 612 had infants who were normal. Exposure to chlorination by-products was assessed by a questionnaire on the mother's habits during pregnancy and by water sampling directly at the mother's home. Levels of trihalomethanes were low (median 1.1 µg/l), whereas chlorate and chlorite levels were relatively high (median 217 µg/l for chlorite and 77 µg/l for chlorate). The authors reported no association of preterm babies with chlorination by-products, but noted that there was a suggested dose–response relationship with small for gestational age. However, the results were not statistically significant (lower confidence intervals below 1.0), and inhalation exposure was also involved; therefore, the results of this study cannot be considered evidence of an effect of dietary exposure to chlorite (Aggazzotti et al., 2004).

3. DIETARY EXPOSURE

3.1 Sources other than food additives

No known natural occurrences have been identified for sodium chlorite, but it is used commercially in substantial amounts in the production of chlorine dioxide for various applications (e.g. bleaching textiles, disinfection and pulp and paper processing) and is also a recognized water disinfection by-product (International Agency for Research on Cancer, 1991).

Chlorite occurs in drinking-water at concentrations ranging from 3.2 to 7.0 mg/l when chlorine dioxide is used for disinfection purposes (World Health Organization, 2003). Chlorine dioxide, chlorite and chlorate may occur in foodstuffs

as a result of their use in flour processing, as a decolorizing agent for carotenoids and other natural pigments (chlorine dioxide), as a bleaching agent in the preparation of modified food starch (sodium chlorite), as an indirect additive in paper and paperboard products used for food packaging (sodium chlorite) and as a defoliant, desiccant and fungicide in agriculture (sodium chlorate).

3.2 Information related to residues in food products treated with acidified sodium chlorite

Depending on the food application, the ASC solution is characterized by a sodium chlorite concentration in the range of 50–150 mg/l and a pH of 2.8–3.2 or 500–1200 mg/l and a pH of 2.5–2.9.

ASC solutions are applied to food surfaces to reduce the number of microbial contaminants. ASC is produced by the addition of a food-grade acid (e.g. citric acid, phosphoric acid, hydrochloric acid, malic acid or sodium hydrogen sulfate) to an aqueous solution of sodium chlorite. Combining the acid with the sodium chlorite solution results in the partial conversion of chlorite to chlorous acid, which is further degraded to chlorite, chlorate, chlorine dioxide and chloride.

The level of formation of chlorous acid, which is considered to be the primary antimicrobial species in ASC solution, depends on the pH of the solution. It exerts its antimicrobial activity by direct disruption of the cellular membrane, as well as by oxidation of cellular constituents. The antimicrobial properties of ASC are also related to the presence of chlorine dioxide in the solution, which forms at low levels as one of the degradation products.

When ASC solution is used for different applications, the highest potential formation of chlorous acid from ASC solution attained at sodium chlorite concentrations between 150 and 1200 mg/l is considered to be between 30% at pH 2.3 and 6% at pH 3.2 (European Food Safety Authority, 2005; Ecolab Inc., 2006).

The potential formation of chlorine dioxide is considered limited and in any case does not exceed 1–3 mg/l (United States Department of Agriculture, 2002; Food Safety Australia New Zealand, 2003; European Food Safety Authority, 2005; Ecolab Inc., 2006). Although levels of chlorine dioxide have been shown to increase during solution ageing, chlorine dioxide is a very volatile compound and is considered not to be present as a residue of the treated food product if ASC from a spray or a dip solution is used according to good manufacturing practices (Ecolab Inc., 2006).

3.3 Overseas food categories and use levels

ASC is intended for use primarily as a spray or as a dipping solution for poultry, meats, vegetables, fruits and seafood, but it can also be used in chilling water. At a pH in the range of 2.8–3.2, chlorous acid (5–35%) is the main active ingredient produced by the reaction and is in equilibrium with hydrogen and chlorite ions (65–95%).

When used in a spray or dip solution, ASC is used at levels that result in sodium chlorite concentrations of 500–1200 mg/l in combination with any acid

approved for use in foods at levels sufficient to provide solutions with pH values in the range 2.5–2.9.

When used in a pre-chiller or chiller water solution, the ASC is used at levels that result in sodium chlorite concentrations of 50–150 mg/l in combination with any acid approved for use in foods at levels sufficient to provide solutions with pH values in the range 2.8–3.2.

3.3.1 Food categories and use levels actually regulated

(a) European Union

Regulation (EC) No. 853/2004 of the European Parliament and of the European Council provides a legal basis permitting the use of antimicrobial treatments to remove surface contamination from poultry. Following a risk assessment conducted by the European Food Safety Authority (EFSA) in 2005 (European Food Safety Authority, 2005), the European Commission (EC) has provided a draft regulation on the approval of substances, including ASC, that can be used to remove surface contamination from fresh poultry carcasses (European Commission, 2006).

(b) USA

The United States regulation, approved under United States Code of Federal Regulations, Title 21, Part 173, section 173.325 (2006), permits a range of ASC solutions of 500–1200 mg sodium chlorite/l at a pH of 2.3–2.9 for food use for poultry meats, red meats, processed, comminuted or formed meat products, intact fruits and vegetables and processed fruit and vegetables, as well as in water and ice that are used to rinse, wash, transport or store seafood at 50 mg sodium chlorite/l (United States Food and Drug Administration, 2006).

(c) Canada

Meat Hygiene Directive 2001-27, 24 May 2001, approves the use of ASC solution at levels of 500–1200 mg sodium chlorite/l at a pH of 2.5–2.9 for use on poultry.

(d) Australia

ASC solution is registered for use at 50–150 mg sodium chlorite/l for whole carcass of poultry and at 500–1200 mg sodium chlorite/l for carcass parts of poultry, meats and formed meats (such as sausages, luncheon meats and pressed hams), fruit and vegetables (intact and cut up), freshwater fish and seafood.

3.3.2 Food categories and use levels proposed by the sponsor

The intended food uses and use levels for ASC proposed in the submission are presented in Table 8. ASC is intended to be applied at use levels of:

- 50–150 mg ASC/l in chilling solution for immersion of whole carcass of poultry (pH 2.8–3.2);
- 500–1200 mg ASC/l for whole carcass or parts of poultry and meats and formed meats (e.g. sausages, luncheon meats, pressed hams) (pH 2.5–2.9); and
- 500–1200 mg ASC/l for fruits and vegetables (whole and sliced), freshwater fish, seafood and eggs (pH 2.5–2.9).

3.4 Residue evaluation of chlorite and chlorate

A number of studies have been conducted to determine residual levels of chlorite and chlorate following treatment with ASC solutions applied under different conditions for the following food categories that are proposed to be treated by the processing aid: poultry carcass, chicken breast, red meat (steak, processed, comminuted or formed), seafood and freshwater fish (fresh salmon, catfish, scallops and shrimps) and fruits and vegetables (whole apple, orange, strawberries, carrot and cucumber, lettuce) (Ecolab Inc., 2006).

3.4.1 Poultry meat

The levels of chlorite and chlorate ions were determined under maximized treatment conditions. This was a pre-chill study consisting of immersion for 5 s in ASC at 1200 mg sodium chlorite/l, pH 2.5, on the wet carcasses after removal from a chiller tank and after 5 min post-removal drip period. The levels of chlorite and chlorate in the carcasses were 9 and 11 µg/kg carcass, respectively. When carcasses were submitted to a 15-or 30-s dip in ASC at 1200 mg sodium chlorite/l, with or without post-treatment water rinsing, the levels of chlorate and chlorite ions remained below 0.1 mg/kg carcass.

Data on the fate of any residual chlorite or chlorate ions on poultry carcasses following removal from the commercial chiller water process (chiller study), consisting of 1 h of immersion in ASC at 150 mg sodium chlorite/l at pH 2.8, were also available. Immediately after ASC application, the levels of chlorite and chlorate detected in the poultry carcasses were 0.54 mg/kg carcass and <0.019 mg/kg carcass, respectively. The levels of chlorite and chlorate were also determined in carcasses and chicken breasts up to 8 h after the treatment. The residual levels of both chlorite and chlorate in the poultry carcasses were below the detection limit of 0.1 mg/kg (European Food Safety Authority, 2005; Ecolab Inc., 2006).

3.4.2 Red meat

The levels of chlorite and chlorate were determined in red meat steak under maximized treatment conditions. This was a pre-chill study consisting of ASC spray for 5–15 s at 1200 mg sodium chlorite/l and pH 2.5, applied on the red meat steak, followed by a 30-s post-removal drip period and by a 1- to 2-s water rinse or air dry. The levels of chlorite and chlorate in the red meat steak remained below 0.1 mg/kg and 0.22 mg/kg steak (levels detected in both test and control samples), respectively, immediately after treatment. When processed, comminuted or formed meats (hot dog) were submitted to an ASC spray or a dip of 1200 mg sodium chlorite/l

Table 8. Summary of the individual proposed food uses and use levels for ASC (expressed as sodium chlorite)

Food category[a]	Food use[a]		Use levels (mg/l)
04.0 Fruits and vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweeds, nuts and seeds	04.1.1.1	Untreated fresh fruit	500–1200
	04.1.1.2	Surface-treated fresh fruit	
	04.1.1.3	Peeled or cut fresh fruit	
	04.2.1.1	Untreated fresh vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes [including soybeans] and aloe vera), seaweeds, nuts and seeds	
	04.2.1.2	Surface-treated fresh vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes and aloe vera), seaweeds, nuts and seeds	
	04.2.1.3	Peeled, cut or shredded fresh vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes and aloe vera), seaweeds, nuts and seeds	
08.0 Meat and meat products, including poultry and game	08.1.1	Fresh meat, poultry and game, whole pieces or cuts	500–1200[b]
	08.1.2	Fresh meat, poultry and game, comminuted	500–1200
09.0 Fish and fish products, including molluscs, crustaceans and echinoderms	09.1.1	Fresh fish and fish products	500–1200
	09.1.2	Fresh molluscs, crustaceans and echinoderms	500–1200
10.0 Eggs and egg products	10.1	Fresh eggs	500–1200

[a] Food category system (Annex B) of the General Standard for Food Additives of the Codex Alimentarius Commission (CODEX STAN 192-1995 [Rev. 6-2005]) (Codex Alimentarius Commission, 2005).

[b] 50–150 mg/l for whole poultry carcasses.

at pH 2.5 for 30 s, with post-treatment water rinsing or air drying, the levels of both chlorite and chlorate were below 0.045 mg/kg immediately after ASC application. The same residual levels of chlorite and chlorate were detected in cooked and uncooked samples up to 24 h after the treatment (Ecolab Inc., 2006).

3.4.3 Seafood and fish

In a study consisting of immersion for 15 s in ASC at 1200 mg sodium chlorite/l, pH 2.3, the levels of chlorite and chlorate were determined on fresh salmon, catfish, scallops and shrimps after a 30-s post-removal drip period with or without water rinse. The levels of chlorite and chlorate were 1.7–38.1 mg/kg and <0.1 mg/kg, respectively. Up to 24–48 h after the treatment, the levels of chlorite and chlorate in treated seafood were below 0.01 mg/kg and 0.1 mg/kg, respectively (Ecolab Inc., 2006).

3.4.4 Fruits and vegetables

Poultry, fresh meat and meat products, seafood and fish are generally not rinsed following application of ASC solution, but a water rinse follows ASC treatment in the case of fruits and vegetables.

Chlorite and chlorate residues were detected on pre-processed produce (0.8 and 0.5 mg/kg, respectively) that was immersed in water following ASC spray treatment for 5–10 s at 1200 mg sodium chlorite/l and pH 2.5. Residues fell to below the limit of detection of 0.1 mg/kg when a high-volume wash was applied for 30 s. In another study, conducted with various types of processed (cut up/sliced) fruits and vegetables (i.e. carrots, melons, lettuce, strawberries, onions and french fries) dipped in or sprayed with ASC at 1200 mg sodium chlorite/l, pH 2.5, for 30 s, then rinsed and held for 24 h, chlorite was detected on many of the unrinsed treated samples. However, a post-treatment wash reduced chlorite levels to below the limit of detection of 0.01 mg/kg in all cases except for ASC-dipped (but not sprayed) lettuce, on which chlorite was detected at 0.23 mg/kg. In all cases, chlorate was below 0.01 mg/kg when the produce was held for 24 h after treatment.

3.4.5 Eggs

No experimental data were provided for residual concentrations of chlorite and chlorate after treatment of eggs with ASC. Therefore, the Committee decided not to take into account this proposed food application in the assessment of ASC-treated food.

3.5 Assessment of dietary exposure

The Committee estimated potential exposure on the basis of the residual concentrations of chlorite and chlorate, as reported in the submitted data for raw products of three food categories (meat and meat products, including poultry; fish and fish products; and fruits and vegetables) that had been treated with ASC solution. The treatment was at the proposed use level of 1200 mg sodium chlorite/l and under optimum conditions to fulfil the technological purpose (with sufficient time of spray or immersion and drip with water wash and holding time). The available data showed that residues of chlorite and chlorate in most foods treated with ASC declined to levels below the limits of detection with time (after treatment, rinsing and a holding period).

The occurrence data used in the calculation of dietary exposure estimates were as follows: for meat and meat products, 0.1 mg/kg for both chlorite and chlorate; for seafood and freshwater fish, 0.01 mg chlorite/kg and 0.1 mg chlorate/kg; for fruits and vegetables, 0.01 mg chlorite/kg for all fruits and vegetables except for leafy vegetables (0.23 mg chlorite/kg) and 0.01 mg chlorate/kg.

Dietary exposures were then estimated using the 13 Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme (GEMS/Food) Consumption Cluster Diets (World Health Organization, 2007) and food consumption data for the general European Union (EU) population based on data of three Member States (Italy, France and Sweden) from the draft EU concise food consumption database, which is currently being developed by EFSA.

The Committee noted that the estimates were highly conservative, as it was assumed that all the treated foods would be consumed daily over a lifetime and that all treated foods consumed contain the maximum residual levels of chlorite and chlorate.

3.5.1 Assessment of per capita dietary exposure based on data from food balance sheets

Dietary exposure assessments per capita are described in Table 9. Average daily estimates of intakes of chlorite and chlorate ranged from 0.2 to 0.7 µg/kg bw and from 0.1 to 0.6 µg/kg bw, respectively. The use of food balance sheets may give an overestimate of average exposure, but underestimate intake for individuals with high intakes of food additives. For this reason, further assessments were conducted, based on individual food consumption data.

3.5.2 Assessment based on individual dietary records

In the EU, an exposure assessment was performed by EFSA in 2005 for the treatment of poultry carcasses with four antimicrobial agents, including ASC (European Food Safety Authority, 2005). An estimate of exposure to chlorite and chlorate was made for the sixty-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) based on EU food consumption of the three food categories from the draft EU concise food consumption database, which is currently being developed by EFSA. This database compiles mean and high percentiles of consumption for about 16 broad food categories from at least three European countries. Mean and high consumptions by adults of meat and meat products (including offal), fruits and vegetables and fish and fish products were extracted from the three national food consumption surveys currently considered—namely, Italy (Turrini et al., 2001), France (Volatier, 2000) and Sweden (Barbieri Becker et al., 2002), which are based on 7-day records for individuals. These figures provide a conservative estimate of mean and high consumption of ASC-treated foods in Europe. They were combined with the data on chlorite and chlorate residues at the proposed use levels to derive dietary exposure estimates for adults in the three reported countries and for children only from France (tables not reported in this monograph).

Table 9. Estimates of per capita dietary exposure to chlorite and chlorate from ASC-treated food, based on 13 GEMS/Food Consumption Cluster Diets

	Per capita dietary exposure (µg/kg bw per day)												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Chlorites													
Fruits and vegetables, except leafy vegetables	0.08	0.16	0.09	0.07	0.10	0.06	0.06	0.08	0.05	0.05	0.10	0.08	0.10
Leafy vegetables	0.02	0.17	0.04	0.10	0.07	0.15	0.16	0.05	0.05	0.04	0.02	0.19	0.15
Meat and meat products	0.07	0.32	0.13	0.15	0.27	0.28	0.13	0.39	0.10	0.06	0.39	0.16	0.47
Fish and fish products	0.002	0.011	0.002	0.004	0.005	0.008	0.008	0.003	0.002	0.003	0.002	0.012	0.004
Total[a]	**0.17**	**0.66**	**0.26**	**0.33**	**0.45**	**0.50**	**0.36**	**0.52**	**0.20**	**0.15**	**0.51**	**0.44**	**0.73**
Chlorates													
Fruits and vegetables, except leafy vegetables	0.08	0.16	0.09	0.07	0.10	0.06	0.06	0.08	0.05	0.05	0.10	0.08	0.10
Meat and meat products	0.07	0.32	0.13	0.15	0.27	0.28	0.13	0.39	0.10	0.06	0.39	0.16	0.47
Fish and fish products	0.024	0.112	0.024	0.038	0.051	0.082	0.079	0.028	0.020	0.027	0.023	0.119	0.041
Total[a]	**0.17**	**0.59**	**0.24**	**0.26**	**0.42**	**0.42**	**0.27**	**0.50**	**0.17**	**0.14**	**0.51**	**0.36**	**0.61**

[a] Sum of the average of potential intake reported for three food categories and assuming a 60-kg average body weight.

Table 10. Estimated dietary exposure of the adult population in Sweden, France and Italy to chlorite and chlorate from ASC-treated food

	Dietary exposure (µg/kg bw per day)					
	Italy		France		Sweden	
	Mean	95th percentile	Mean	95th percentile	Mean	95th percentile
Chlorite						
Fruits and vegetables[a]	1.72	2.71	0.99	2.26	0.69	1.48
Meat and meat products	0.23	0.44	0.23	0.44	0.18	0.42
Fish and fish products	0.01	0.02	0.01	0.02	0.00	0.01
Total[b]	**1.95**	**2.71**	**1.22**	**2.26**	**0.88**	**1.48**
Chlorate						
Fruits and vegetables[a]	0.07	0.12	0.04	0.10	0.03	0.06
Meat and meat products	0.23	0.44	0.23	0.44	0.18	0.42
Fish and fish products	0.07	0.20	0.06	0.16	0.04	0.14
Total[b]	**0.37**	**0.44**	**0.33**	**0.44**	**0.26**	**0.42**

[a] Sum of the highest average consumption of fruits or vegetables plus the highest 95th percentile consumption of fruits or vegetables only for Italy and Sweden has been used in this assessment.

[b] Sum of the average and the highest 95th percentiles of potential dietary exposure reported from a single food group from three food categories and assuming a 60-kg average body weight for adults.

The highest total estimates of daily exposure in the adult EU population, if a 60-kg average body weight is assumed, were 0.9 µg/kg bw (mean) and 2.7 µg/kg bw (95th percentile) for chlorite and 0.3 µg/kg bw (mean) and 0.4 µg/kg bw (95th percentile) for chlorate (Table 10). In the child population (France, aged 3–14 years, tables not shown), if a 30-kg average body weight is assumed, total estimates of daily exposure were 1.6 µg/kg bw (mean) and 2.8 µg/kg bw (95th percentile) for chlorite and 0.5 µg/kg bw (mean) and 0.6 µg/kg bw (95th percentile) for chlorate.

4. COMMENTS

4.1 Toxicological data

The available database to assess the safety of ASC and its residues chlorite and chlorate has limitations, and few of the studies have been conducted to the current standards expected for regulatory submissions. The studies on ASC related to a germicidal product, and some of these involved parenteral administration. The Committee considered that although not directly relevant to oral exposure, these studies provided useful supplementary information that did not raise concern about the use of acidified chlorite as a processing aid. In many of the studies in which chlorite or chlorate was administered in drinking-water, the information provided was insufficient to derive an accurate estimation of the dose received by the animals.

Chlorite and chlorate are rapidly absorbed into the plasma and distributed throughout the body, with the highest concentrations in plasma. They are excreted primarily in the urine in the form of chloride, with lesser amounts of chlorite and chlorate. However, the extent to which these are formed as chemical degradation products prior to absorption or as a result of biotransformation was unclear. There was some indication of metabolism to chloroform, but the data were inadequate to evaluate or to use in the safety assessment.

ASC and chlorite are of moderate acute toxicity, but only limited data were available on chlorate. Studies conducted with sodium chlorite in a number of species demonstrated that the most consistent finding is oxidative stress associated with changes in erythrocytes. This observation was also supported by a number of biochemical studies conducted in vitro. Some studies have indicated that the effect may be related to a reduction in serum GSH levels, thus reducing the body's ability to protect the erythrocytes from the effects of sodium chlorite. Other studies have indicated that sodium chlorite may cause damage to the erythrocyte membrane. For effects on erythrocytes, the lowest LOAEL of 19 mg/kg bw per day, expressed as chlorite, was derived from a 13-week gavage study in rats in which the NOAEL was 7.4 mg/kg bw per day, expressed as chlorite. Studies on sodium chlorate in a number of species showed some effects on haematological parameters and on body weight gain.

Although sodium chlorate has also been reported to have effects on erythrocytes, changes in thyroid histology (e.g. colloid depletion, hypertrophy, incidence and severity of hyperplasia) and in thyroid hormones were the most sensitive effects observed in rats administered sodium chlorate in drinking-water for 21 or 90 days. Male rats were more sensitive than females, as is commonly seen with substances that affect thyroid function. In one of the two available 90-day studies, thyroid hypertrophy and decreased colloid were observed in male rats given sodium chlorate at drinking-water concentrations of 1 mg/l as chlorate (equivalent to about 0.1 mg chlorate/kg bw per day) and above. In general, effects including incidence and severity of follicular cell hyperplasia were dose related and more consistently observed at chlorate doses of 75 mg/kg bw per day and above.

Sodium chlorite was not carcinogenic following a number of long-term studies, although these were not conducted to current standards. The International

Agency for Research on Cancer concluded in 1991 that sodium chlorite was not classifiable with respect to carcinogenicity to humans. Sodium chlorite has given positive results in some, but not all, in vitro genotoxicity assays and in one of the two available in vivo mouse micronucleus assays involving intraperitoneal administration. Negative results were obtained in several in vivo assays, for induction of bone marrow micronuclei, chromosomal aberrations and sperm head abnormalities, involving oral administration of sodium chlorite to mice.

Sodium chlorate has recently been tested for carcinogenicity in rats and mice under the United States National Toxicology Program; results of these studies were not available at the time WHO set the TDI for chlorate. There was no evidence of carcinogenic activity in male B6C3F1 mice and equivocal evidence in female mice based on marginally increased incidences of pancreatic islet neoplasms. Sodium chlorate produced positive results in some in vitro assays, but not for induction of bone marrow micronuclei or chromosomal aberrations following oral administration to mice. There was some evidence of carcinogenic activity in male and female F344/N rats based on increased incidences of thyroid gland neoplasms. The incidence of thyroid gland follicular hypertrophy was enhanced compared with control groups at doses lower than those resulting in increased tumour incidences and was significantly greater than control in the male rats at all tested doses. Therefore, the lowest dose, equivalent to approximately 5 mg/kg bw per day, expressed as chlorate, was the LOAEL. Because a NOAEL was not identified in the study, the Committee decided to apply a BMD approach to derive a point of departure on the dose–response curve (see Annex 1, reference *176*). The USEPA BMD software version 1.4.1 was used for modelling the rat thyroid gland follicular cell hypertrophy data. The calculated BMD_{10} values for a 10% increase in thyroid gland follicular cell hypertrophy in the male rats ranged from 1.9 to 5.9 mg/kg bw per day, expressed as chlorate. The values of the 95% lower confidence limit for the BMD ($BMDL_{10}$) ranged from 1.1 to 4.4 mg/kg bw per day, expressed as chlorate. The Committee used the lowest $BMDL_{10}$ of 1.1 mg/kg bw per day, expressed as chlorate, which was derived from the model giving the best fit to the data, for its further evaluation of chlorate. For female rats, the BMD_{10} values ranged from 4.7 to 12.6 mg/kg bw per day, and the $BMDL_{10}$ values ranged from 3.0 to 6.4 mg/kg bw per day.

Based on the negative in vivo genotoxicity data and the nature of the histopathological observations, the Committee concluded that a non-genotoxic mode of action was likely for the induction of thyroid tumours by sodium chlorate. This mode of action is likely to be mediated via decreased serum thyroid hormones, leading to increased release of TSH and consequent stimulation of thyroid cell proliferation and thyroid gland growth, which can lead to thyroid tumours in rodents.

In addition to thyroid carcinogenesis, this mode of action raises concerns about possible neurodevelopmental effects, since thyroid hormone status is critical to normal brain development.

Reproductive toxicity studies have shown no adverse effects of ASC or sodium chlorite on fertility. A multigeneration study of reproduction and developmental neurotoxicity was available in which sodium chlorite was administered to rats in drinking-water at a concentration of 35, 70 or 300 mg/l.

Published information indicated that the highest dose tested resulted in effects on body weight in both sexes of the parental generation and a range of effects in the offspring, including decreased body weight, changes in haematological parameters and a decrease in maximum startle response amplitude at PND 24, but not at PND 60. A small but statistically significant decrease in maximum startle response amplitude was also reported at the middle dose at PND 24. The Committee considered that this observation was attributable to perturbed habituation in the control animals. Other effects observed in the offspring of the high-dose group (i.e. reduced absolute brain weight and slight delays in attainment of sexual maturity) could be attributable to reduced body weight. In addition, a USEPA toxicological review of chlorine dioxide and chlorite cited data contained only in the unpublished original study report showing reduced absolute and relative liver weights in the F_0 females and F_1 males and females of the high-dose group and in the F_0 females and F_1 males of the mid-dose group. The Committee concluded that the low dose in this study, equivalent to 3 mg/kg bw per day, expressed as chlorite, was the NOAEL.

Administration of sodium chlorate to pregnant rats resulted in no maternal or developmental effects at the highest tested dose of 1000 mg/kg bw per day. Neurodevelopmental end-points were not investigated in this study, and no multigeneration study was available.

Special studies on nephrotoxicity, immune function and sperm quality in vivo indicated that such effects would not be critical to the safety assessment.

Studies in healthy adult male volunteers lasting up to 12 weeks showed no clear treatment-related effects on blood, urinalysis or physical examination at doses of sodium chlorite and sodium chlorate estimated to be in the region of 0.036 mg/kg bw per day, expressed as chlorite or chlorate.

4.2 Assessment of dietary exposure

The Committee estimated potential dietary exposure on the basis of the residual concentrations of chlorite and chlorate, as reported in the submitted data for raw products of three food categories (meat and meat products, including poultry; fish and fish products; and fruits and vegetables) that had been treated with ASC solution. The treatment was at the proposed use level of 1200 mg sodium chlorite/l and under optimum conditions to fulfil the technological purpose (with sufficient time of spray or immersion and drip with water wash and holding time).

The available data showed that residues of chlorite and chlorate in most foods treated with ASC declined to levels below the limits of detection with time (after treatment, rinsing and a holding period).

The occurrence data used in the calculation of dietary exposure estimates were as follows: for meat and meat products, 0.1 mg/kg for both chlorite and chlorate; for seafood and freshwater fish, 0.01 mg chlorite/kg and 0.1 mg chlorate/kg; for fruits and vegetables, 0.01 mg chlorite/kg for all fruits and vegetables, except for leafy vegetables (0.23 mg chlorite/kg), and 0.01 mg chlorate/kg.

Dietary exposures were then estimated using the 13 GEMS/Food Consumption Cluster Diets and food consumption data from the EU countries for the general population. The Committee noted that the estimates were highly conservative, as it was assumed that all the treated foods would be consumed daily over a lifetime and that all treated foods consumed contain the maximum residual levels of chlorite and chlorate.

International mean dietary exposures were estimated to be 0.2–0.7 µg/kg bw per day for chlorite and 0.1–0.6 µg/kg bw per day for chlorate for the 13 GEMS/Food Consumption Cluster Diets, if a body weight of 60 kg is assumed. National estimates for EU countries of mean to 95th percentile daily dietary exposures in the general population were 0.9–3 µg/kg bw for chlorite and 0.3–0.6 µg/kg bw for chlorate.

5. EVALUATION

The Committee concluded that the available toxicological data were sufficient to assess the safety of ASC by setting ADIs for chlorite and chlorate.

For chlorite, the Committee established an ADI of 0–0.03 mg/kg bw on the basis of the NOAEL of 3 mg/kg bw per day for reduced liver weight of F_0 females and F_1 males and females in a two-generation reproductive study in rats and a safety factor of 100 to allow for inter- and intraspecies variability. This ADI is supported by the results of studies in human volunteers showing no adverse effects at this intake.

For chlorate, the Committee concluded that the most sensitive effects were changes to the thyroid gland of male rats. Rats are considered to be highly sensitive to the effects of agents that disrupt thyroid hormone homeostasis. The Committee considered that humans are likely to be less sensitive than rats to these effects and that a safety factor for interspecies variation was not required. However, the Committee noted deficiencies in the database, particularly with respect to investigation of possible neurodevelopmental effects. The Committee therefore established an ADI of 0–0.01 mg/kg bw for chlorate on the basis of the $BMDL_{10}$ of 1.1 mg/kg bw per day for non-neoplastic effects on the thyroid of male rats in a recent carcinogenicity study, a safety factor of 10 to allow for intraspecies variability and an additional factor of 10 to allow for the deficiencies in the database.

The Committee noted that the occurrence data submitted for chlorite and chlorate, determined using good manufacturing practice for ASC-treated foods, were sufficient to be used in the assessment. These occurrence data were used with national diet data for EU countries and the 13 GEMS/Food Consumption Cluster Diets in a dietary exposure scenario whereby all treated food categories consumed contained chlorite and chlorate at the maximum residual concentrations.

For chlorite, a dietary exposure of 3 µg/kg bw per day could be taken to represent high consumers, including children. For chlorate, a dietary exposure of 0.6 µg/kg bw per day could be taken to represent high consumers, including children.

The Committee concluded that the present conservative estimates of mean and high-level dietary exposure to chlorite and chlorate represented up to 10% of the ADIs. The Committee noted that these estimates were compatible with the exposure allocated to other sources within the WHO drinking-water guidelines for chlorite and chlorate.

Because ASC is a surface treatment, the residue level will be proportional to the surface/volume ratio. Therefore, the current evaluation relies on the submitted protocols, and dietary exposure should be re-estimated if new usages are introduced (e.g. ground beef).

The Committee noted that the use of ASC does not replace the need for good hygienic practices in handling and processing of food.

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ASPARAGINASE FROM ASPERGILLUS ORYZAE *EXPRESSED IN* ASPERGILLUS ORYZAE

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1. EXPLANATION

At the request of the Codex Committee on Food Additives and Contaminants (Codex Alimentarius Commission, 2006) at its thirty-eighth session, the Committee evaluated an enzyme preparation containing the enzyme asparaginase (L-asparagine amidohydrolase; EC 3.5.1.1), which it had not evaluated previously. Asparaginase hydrolyses the amide in the amino acid L-asparagine to the corresponding acid, resulting in L-aspartate (aspartic acid) and ammonia. Apart from asparagine, asparaginase acts only on glutamine and has no activity on other amino acids. Asparaginase has no activity on asparagine residues in peptides or proteins.

The asparaginase enzyme preparation under consideration is produced by submerged fermentation of an *Aspergillus oryzae* production strain carrying a gene encoding asparaginase from *A. oryzae*. The enzyme is subsequently partially purified and concentrated, resulting in a liquid enzyme concentrate (LEC), which, in the final preparation, is stabilized, formulated and standardized with water, glycerol, sodium benzoate and potassium sorbate.

The enzyme activity of asparaginase is expressed in asparaginase units (ASNU), 1 ASNU being the amount of enzyme that produces 1 µmol of ammonia per minute under specific reaction conditions. The asparaginase preparation has a typical activity of 3500 ASNU/g and has the following composition: total organic solids (TOS), approximately 4%; water, approximately 46%; glycerol, approximately 50%; sodium benzoate, approximately 0.3%; and potassium sorbate, approximately 0.1%.

Asparaginase is to be used during food manufacture to convert asparagine to aspartic acid with the intention of reducing acrylamide formation during food production of dough-based products, such as cookies and crackers, and processed potato products, such as potato chips and french fries. Acrylamide is formed as a reaction product between asparagine and reducing sugars when food products are baked or fried at temperatures above 120 °C. The enzyme preparation is added to dough prior to baking, at recommended use levels varying from 200 up to 2500 ASNU (or 0.06–0.7 g of the enzyme preparation) per kilogram of processed food. Prior to the heat treatment or cooking step, potato strips or slices are treated by dipping in a water bath containing the enzyme preparation, leading up to approximately 2000 ASNU (or 0.6 g) per kilogram of product.

1.1 Genetic modification

The host strain for the asparaginase gene, the *Aspergillus oryzae* BECh2 strain, was derived from *A. oryzae* strain IFO 4177 (synonym A1560). *Aspergillus oryzae* is known to contain genes involved in the synthesis of the secondary metabolites cyclopiazonic acid, kojic acid and 3-β-nitropropionic acid, as well as genes involved in the synthesis of aflatoxins. In a first step, *A. oryzae* strain A1560 was genetically modified by site-directed disruption of the endogenous amylase and protease genes to allow the production of asparaginase without enzymatic side activities. In the following two steps, the modified strain (designated *A. oryzae* JaL 228) was irradiated to remove or reduce its potential to produce secondary metabolites. First, the JaL 228 strain was exposed to γ-radiation, resulting in a mutant (designated *A. oryzae* BECh1) that is devoid of genes involved in the synthesis of aflatoxins and cyclopiazonic acid. Subsequently, the BECh1 strain was subjected to ultraviolet radiation, resulting in a mutant (designated *A. oryzae* BECh2) that is impaired in kojic acid synthesis. It is this BECh2 strain that is used as the host strain for the asparaginase gene. When tested under conditions optimal for the production of secondary metabolites, the BECh2 strain produced neither aflatoxins (including the intermediate compounds sterigmatocystin and 5-methoxy-sterigmatocystin) nor cyclopiazonic acid and essentially no 3-β-nitropropionic acid. Although the strain produced kojic acid, it did so only at a level of approximately 15% of that produced by the A1560 and BECh1 strains.

The asparaginase gene originates from the same strain as the host strain, i.e. *A. oryzae* strain IFO 4177 (synonym A1560), and is cloned into an *A. oryzae* expression plasmid generating the asparaginase expression plasmid pCaHj621. This expression plasmid is based on the standard *Escherichia coli* vector pUC19 and contains known and well characterized deoxyribonucleic acid (DNA) sequences. The pCaHj621 expression plasmid was used to transform the *A. oryzae* BECh2 host strain to obtain the *A. oryzae* pCaHj621/BECh2#10 production strain. The plasmid is stably integrated into the *A. oryzae* chromosomal DNA and does not contain antibiotic resistance genes. The inserted DNA also does not encode or express any known harmful or toxic substances. Asparaginase expressed by the production strain has no significant amino acid sequence homology with known allergens and toxins listed in publicly available databases. When one test batch (PPV 24743) of the enzyme preparation was analysed for kojic acid and 3-β-nitropropionic acid, these secondary metabolites were not detected.

1.2 Product characterization

Asparaginase is produced by submerged fed-batch pure culture fermentation of the *A. oryzae* pCaHj621/BECh2#10 production strain. It is secreted into the fermentation medium, from which it is recovered and concentrated. It is subsequently stabilized, formulated and standardized with water, glycerol, sodium benzoate and potassium sorbate. The enzyme preparation is added to dough prior to baking, and potatoes are dipped in water containing the enzyme preparation before frying. During the heating of the products, it is expected that the enzyme will be inactivated.

The asparaginase enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing, prepared by the Committee at its sixty-seventh meeting (Annex 1, reference *184*). The enzyme preparation is free from the production organism.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

Aspergillus oryzae asparaginase was assessed for potential allergenicity by comparing its amino acid sequence with those of known allergens listed in publicly available databases (SWALL and GenBank). No immunologically significant sequence homology was detected. A sequence homology assessment of *A. oryzae* asparaginase with respect to the sequences of known toxins listed in the same databases also revealed no significant homology.

2.2 Toxicological studies

The host organism *A. oryzae* is non-pathogenic and has a long history of use in food. Enzyme preparations from *A. oryzae* have been evaluated previously by the Committee. For α-amylase and protease from *A. oryzae*, the Committee concluded that, since they are derived from a microorganism that is accepted

as a constituent of foods and is normally used in food production, they must be regarded as foods and are thus acceptable for use in food processing (Annex 1, reference *77*). An acceptable daily intake (ADI) "not specified" was allocated to lipase from *A. oryzae* (Annex 1, reference *35*), as well as to laccase from a recombinant strain of *A. oryzae* (Annex 1, reference *167*). Phospholipase A1 from a recombinant strain of *A. oryzae*, which was evaluated at the sixty-fifth meeting of the Committee (Annex 1, reference *178*), is again under consideration at the present meeting (see monograph in this volume).

Aspergillus oryzae host strains derived from strain A1560 have been used in the construction of several Novozymes enzyme production strains. The *A. oryzae* BECh2 strain used as host strain in the construction of the asparaginase production strain was also used to develop production strains for triacylglycerol lipase, glucose oxidase, two xylanases and phospholipase A1, which is under consideration at the present meeting (see monograph in this volume). The DNA introduced into the production strains for these enzymes is essentially the same as that introduced into the asparaginase production strain *A. oryzae* pCaHj621/BECh2#10, except for the sequence encoding the specific enzyme. All these enzyme products were stated to have been assessed for safety (in at least a 13-week study of toxicity in rats treated orally, an assay for mutagenicity in bacteria in vitro and a cytogenetic assay in human lymphocytes in vitro).

Toxicological studies were also performed with the asparaginase enzyme, using an LEC (batch PPV 24743; dry matter content, 10.5% weight per weight [w/w]; TOS content, 8.4% w/w; specific gravity, 1.049 g/ml), omitting stabilization, formulation and standardization.

2.2.1 Acute toxicity

No information was available.

2.2.2 Short-term studies of toxicity

Groups of 10 male and 10 female CD rats (aged 42–48 days) were given asparaginase (batch PPV 24743) at a dose equivalent to 0, 88, 290 or 880 mg TOS/kg body weight (bw) by gavage (in purified water) daily for 13 weeks. The study was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (1998) and was certified for compliance with Good Laboratory Practice (GLP) and quality assurance (QA). Observations included clinical signs, physical examination and arena observations, body weight, food intake, water consumption, sensory reactivity, grip strength, motor activity, ophthalmoscopy, haematology, clinical chemistry, organ weights and macroscopic and microscopic pathology.

No treatment-related effects were observed on mortality, clinical signs, ophthalmoscopy, body weight, food conversion efficiency, sensory reactivity or motor activity. Forelimb and hindlimb grip strength were slightly increased (12%) in males at the mid and high doses, but there was no dose–response relationship, and statistical significance was reached only for forelimb grip strength at the high dose. Treated males also showed decreases in haematocrit, haemoglobin, red blood cell

count and total and differential white blood cell counts. Only for haematocrit and haemoglobin at the mid and high doses did the difference reach statistical significance; however, the difference was very small (4%), without a dose–response relationship. Treated females showed a small decrease in food consumption (7% at the high dose) and a small increase in water consumption (8% and 13% at the mid and high doses, respectively), but no effect on food conversion efficiency. Treated females also showed statistically significant decreases in total white blood cell and basophil counts and in activated partial thromboplastin time. However, there was no dose–response relationship for any of the changed parameters, and control values were high when compared with historical control values for these parameters. In both sexes, organ weights, macroscropic pathology and histopathology were unaffected by treatment. The only treatment-related findings were small (7–8%) but statistically significant increases in plasma potassium levels in males at the mid and high doses and in females at the high dose. As there were no other changes in electrolyte concentrations and the kidneys appeared histopathologically normal, these changes were considered to be of no toxicological significance. Overall, it can be concluded that the no-observed-effect level (NOEL) is 880 mg TOS/kg bw per day, the highest dose tested in this study (Hughes, 2006).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.2.4 Genotoxicity

The results of two studies of genotoxicity with asparaginase (batch PPV 24743) in vitro are summarized in Table 1. The first study followed OECD Test Guideline 471 (1997), and the second, OECD Test Guideline 473 (1997). Both studies were certified for compliance with GLP and QA.

2.2.5 Reproductive toxicity

No information was available.

2.3 Observations in humans

No information was available.

3. DIETARY EXPOSURE

The asparaginase enzyme preparation is added to dough prior to baking, and potatoes are dipped in water containing the enzyme preparation before frying. Although it is expected that the enzyme will be inactivated upon heating of the products, the actual levels of the enzyme (active or inactive) in the final food products are not known. A worst-case scenario for human dietary exposure can be estimated on the basis of the recommended use levels and the assumption that all TOS originating from the enzyme preparation are carried over into the final products. To elaborate this scenario, it is assumed that:

- all cereal and potato products are produced by processes using the asparaginase enzyme preparation at the highest recommended use level;
- a maximum concentration of 700 mg of enzyme preparation (or 2500 ASNU) is applied in treating 1 kg of processed food;
- the TOS content of the enzyme preparation is 4%;
- all TOS are carried over into the final products.

Table 1. Genotoxicity of asparaginase in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrApKM101	156–5000 µg/ml, ±S9	Negative[a]	Pedersen (2006)
Chromosomal aberration	Human lymphocytes	1st experiment: 1187, 2813 or 5000 µg/ml, −S9; 1582, 2109 or 5000 µg/ml, +S9 2nd experiment: 430, 839 or 1311 µg/ml, −S9; 3200, 4000 or 5000 µg/ml, +S9	Negative[b]	Whitwell (2006)

S9, 9000 × *g* supernatant from rat liver.

[a] With and without metabolic activation (S9), by the "treat-and-plate" method (to avoid problems owing to the presence of free amino acids such as histidine and tryptophan in the asparaginase preparation). In the test with *E. coli*, initially strain WP2uvrA was tested by the direct plate incorporation method. However, because the asparaginase preparation supported growth of the test bacteria in this procedure, with a concurrent increase in the number of revertants, the experiment was repeated using the "treat-and-plate" method and the strain WP2uvrApKM101, which was considered more sensitive to pro-mutagens in the presence of S9.

[b] With and without metabolic activation (S9). In the first experiment, the cell cultures were treated for 3 h without and with S9 and were harvested 17 h later. The highest tested concentration induced 44% and 33% mitotic inhibition in the absence and presence of S9, respectively. In the second experiment, the cells were exposed continuously for 20 h without S9 and then harvested. With S9, the cells were treated for 3 h and harvested 17 h later. The highest tested concentration induced 53% and 0% mitotic inhibition in the absence and presence of S9, respectively.

The resulting maximum concentration of enzyme in the final products would correspond to 28 mg TOS/kg of food.

According to the Joint FAO/WHO Expert Committee on Food Additives (JECFA) exposure assessment for acrylamide, the highest amount of total cereals and potato[1] products from the five Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme (GEMS/Food) Regional Diets is 613 g/day (African diet; Table 2). If such a conservative consumption figure is assumed, as well as a body weight of 60 kg, this would result in a dietary exposure to asparaginase of about 17 mg TOS/day or 0.3 mg TOS/kg bw per day.

In order to harmonize the approaches and to follow the recommendations from the FAO/WHO consultation on dietary exposure assessment, another estimate could be based on the 13 GEMS/Food Consumption Cluster Diets (Table 3). According to these data, the maximum consumption for cereals and potato products would be 960 g/day. Based on this figure, the dietary exposure to asparaginase would be 27 mg TOS/day or 0.4 mg TOS/kg bw per day, if a body weight of 60 kg is assumed.

Table 2. Consumption of cereals and roots and tubers from the five GEMS/Food Regional Diets

Commodity	Consumption (g/person per day) from GEMS/Food Regional Diets				
	Middle Eastern	Far Eastern	African	Latin American	European
Total cereals	430	451	292	254	222
Total roots & tubers	62	109	321	159	242
Total	**492**	**560**	**613**	**413**	**464**

Table 3. Consumption of cereals and roots and tubers from the 13 GEMS/Food Consumption Cluster Diets

Commodity	Consumption (g/person per day) from GEMS/Food Consumption Cluster Diets												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Total cereals	357	714	763	505	365	329	617	487	389	386	440	568	410
Total roots & tubers	525	246	69	244	278	205	120	101	404	438	135	104	176
Total	**882**	**960**	**832**	**749**	**643**	**534**	**737**	**588**	**793**	**824**	**575**	**672**	**586**

[1] Represented by the broad category “roots and tubers”.

4. COMMENTS

4.1 Toxicological data

Toxicological studies were performed with an asparaginase LEC. The Committee noted that the materials added to the asparaginase LEC for stabilization, formulation and standardization either have been evaluated previously by the Committee or are common food constituents and do not raise safety concerns.

In a 13-week study of toxicity in rats, no significant treatment-related effects were seen when the LEC was administered by oral gavage at doses up to and including 880 mg TOS/kg bw per day. Therefore, 880 mg TOS/kg bw per day, the highest dose tested, was taken to be the NOEL. The LEC was not mutagenic in an assay for mutagenicity in bacteria in vitro and was not clastogenic in an assay for chromosomal aberrations in mammalian cells in vitro.

4.2 Assessment of dietary exposure

Based on a maximum mean daily consumption of 960 g of processed foods (cereals, roots and tubers; GEMS/Food Consumption Cluster Diet B) by a 60-kg adult and on the assumptions that the enzyme is used at the maximum recommended use level and that all TOS originating from the enzyme preparation remain in the final products, the dietary exposure would be 0.4 mg TOS/kg bw per day.

5. EVALUATION

Comparing the conservative exposure estimate with the NOEL from the 13-week study of oral toxicity, the margin of safety is 2200. The Committee allocated an ADI "not specified" for asparaginase from this recombinant strain of *A. oryzae*, used in the applications specified and in accordance with good manufacturing practice.

6. REFERENCES

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CARRAGEENAN AND PROCESSED EUCHEUMA *SEAWEED (addendum)*

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1. EXPLANATION

Carrageenan is a sulfated galactose polymer with an average molecular weight well above 100 kilodaltons (kDa). It is derived from several species of red seaweeds of the class Rhodophyceae. It has no nutritive value and is used in food preparation for its gelling, thickening and emulsifying properties. The three main copolymers in carrageenan are designated as *iota* (ι), *kappa* (κ) and *lambda* (λ), depending on the number and location of the sulfate moieties on the hexose backbone. Processed *Eucheuma* seaweed is a semi-refined form of carrageenan and has been derived from either *E. cottonii* or *E. spinosum*.

Carrageenan was reviewed by the Committee at its thirteenth, seventeenth, twenty-eighth, fifty-first and fifty-seventh meetings (Annex 1, references *19*, *32*,

66, *137* and *154*). At its twenty-eighth meeting, the Committee established an acceptable daily intake (ADI) "not specified" on the basis of the results of a number of toxicological studies on carrageenans obtained from various sources. Processed *Eucheuma* seaweed was reviewed by the Committee at its thirtieth, thirty-ninth, forty-first, forty-fourth, fifty-first and fifty-seventh meetings (Annex 1, references *73*, *101*, *107*, *116*, *137* and *154*). At its fifty-first meeting, the Committee concluded that the toxicities of processed *Eucheuma* seaweed and carrageenan were sufficiently similar for the ADI "not specified" for carrageenan to be extended to a temporary group ADI including processed *Eucheuma* seaweed, pending clarification of the significance of the promotion of colon cancer observed in experiments in rats. At its fifty-seventh meeting, the Committee established a group ADI "not specified" for the sum of carrageenan and processed *Eucheuma* seaweed, as the Committee considered that the intakes of carrageenan and processed *Eucheuma* seaweed from their use as food additives were of no safety concern.

At its present meeting, the Committee was requested by the Codex Committee on Food Additives and Contaminants to review all data necessary for toxicological re-evaluation, including specific data relevant to the safety assessment for infants aged 0–6 months, from exposure through infant formulas (Codex Alimentarius Commission, 2006a).

At the present meeting, the Committee reviewed data published since the fifty-seventh meeting in 2001 and other data of specific relevance to the safety assessment for infants. In response to the Committee's request for further data, a toxicological dossier on carrageenan and processed *Eucheuma* seaweed was submitted. In addition, a search of the scientific literature was conducted for the years 2000–2007.

The Committee also considered some new information related to the specifications.

2. BIOLOGICAL DATA

2.1 Toxicological studies

2.1.1 Special studies on the gastrointestinal tract

Three groups of five female BALB/c mice were administered drinking-water containing 0 (control), 1% or 4% λ-carrageenan (extracted from *Gigartina acicularis* and *G. pisillata*) continuously for 10 weeks. These concentrations were equal to doses of 0, 1120 and 3520 mg/kg body weight (bw) per day, respectively (based on measured fluid intake for each treatment group). The carrageenan administered was purchased commercially and was not food grade, but was described by the manufacturer as "pure undegraded *lambda* carrageenan". Animals were housed individually in stainless steel cages and had unlimited access to AIN-76 diet. This study assessed pro-inflammatory effects of carrageenan in the mouse colon prior to a series of further experiments investigating combined treatment with *N*-methyl-*N*-nitrosourea (MNU) (see below). At study termination, mice were sacrificed, and histological examination of the colons was conducted

using the "Swiss roll" method. Results were analysed by parametric one-way analysis of variance (ANOVA).

All animals in the water-only treatment group (controls) had solid stools throughout the study. Stools became semi-soft in treated groups after 32 days (1% carrageenan) and 7 days (4% carrageenan). Body weight gain was comparable in all groups. Fluid intake was reduced in the mice receiving carrageenan at 4% but not 1% in the drinking-water. At autopsy, redness was observed in the proximal third of the colon in all carrageenan-treated mice; in three mice treated with 4% carrageenan, redness extended right through the proximal two thirds of the colon. Histological examination of colons of mice treated with 4% carrageenan revealed an increased crypt loss compared with the 1% treatment group ($P = 0.03$) and control ($P = 0.002$). Both carrageenan doses induced hyperplasia ($P < 0.001$) and inflammation ($P < 0.05$) compared with controls. No abnormalities were observed in the water-only treatment group (Donnelly et al., 2004a).

Cohen & Ito (2006) concluded that because the carrageenan used in these experiments was purchased commercially, it probably did not comply with the specifications of food-grade carrageenan. They speculated that it may have had a higher viscosity than that of food-grade carrageenan (the typical viscosity of carrageenan sent to suppliers is 700–800 centipoise), which might have resulted in a reduced availability of free water at concentrations of 1% and 4% carrageenan. However, this view is not supported by the reported body weight and fluid intake data.

In a series of further experiments with the same type of carrageenan, individual and combined effects of λ-carrageenan and MNU were assessed in groups of five female BALB/c mice. A single intraperitoneal injection of MNU (62.5 mg/kg bw) was combined with λ-carrageenan in the drinking-water (1% or 4%) over 1, 3 or 10 weeks. In addition, both MNU (62.5 mg/kg bw) and λ-carrageenan (1% or 4%) were administered alone for 10 weeks. The respective doses (based on measured fluid intake for each treatment group) were equal to 980 and 3920 mg/kg bw per day (7 days); 1070 and 4280 mg/kg bw per day (21 days); and 1090 and 3560 mg/kg bw per day (10 weeks). At study termination, mice were sacrificed, and colons were prepared for histological analysis using the Swiss roll method. Stem cell mutations and mutant clonal expansion were assessed by the metallothionein endogenous reporter gene assay. Statistical analysis was by one-way ANOVA. Combined treatment induced significantly greater crypt stem cell mutations and a significantly greater number and size of aberrant crypt foci than did treatment with MNU or carrageenan alone. Significant effects on metallothionein crypt immunopositivity were observed at all durations of exposure with combined treatment. The authors concluded that undegraded λ-carrageenan enhanced the effect of MNU upon metallothionein crypt immunopositivity in mouse colon (Donnelly et al., 2004a, 2004b, 2004c, 2005).

A study conducted to Good Laboratory Practice (GLP) in accordance with Organisation for Economic Co-operation and Development (OECD) guidelines tested the hypothesis that food-grade κ-carrageenan is safe for food use. The κ-carrageenan (molecular weight 196–257 kDa) was extracted from *E. cottonii*

seaweed and contained an average low molecular weight tail fraction of 7% below 50 kDa. Groups of 20 male and 20 female CDF (F-344)/Crl/BR rats were fed diets containing 25 000 or 50 000 mg carrageenan/kg ad libitum for 90 consecutive days. The mean doses of carrageenan were equal to 1656 ± 331 mg/kg bw per day (males) and 1872 ± 254 mg/kg bw per day (females) for the 25 000 mg/kg diet dose group; and 3394 ± 706 mg/kg bw per day (males) and 3867 ± 647 mg/kg bw per day (females) for the 50 000 mg/kg diet dose group. During the in-life phase, the following parameters were evaluated: mortality, physical examinations, body weights, feed consumption and ophthalmic examinations. Following termination, evaluations of haematology, serum chemistry, urinalysis and organ weights were performed. Necropsy was conducted on all animals from the control and 50 000 mg/kg groups, including histopathological evaluation on an extensive range of organs and examination of the intestinal tissue according to the Swiss roll method. All tissues were fixed in 10% buffered formalin.

There were no treatment-related deaths. Soft faeces and brown material on the anogenital area were observed in the high-dose group and to a limited degree in the low-dose group; however, these findings were not considered adverse by the authors. There was no effect on body weights and no toxicologically significant findings on clinical chemistry investigations, haematological effects or urinalysis parameters. In addition, no treatment-related findings were noted during necropsy or the ophthalmic examination at the study termination. There were no treatment-related effects on absolute organ weights and organ to brain weight ratios. Microscopic examination revealed no treatment-related effects in any of the tissues examined, and there was no evidence of aberrant crypt foci in the colon examinations. The authors concluded that the no-observed-adverse-effect level (NOAEL) was >50 000 mg carrageenan/kg in the diet, equal to 3394 mg/kg bw per day for males and 3867 mg/kg bw per day for females (Weiner et al., 2007).

In a study reviewed by the Committee at its twenty-eighth and fifty-first meetings, an infant formula with added carrageenan was tested in infant baboons (*Papio cynocephalus*) from day 1 until 112 days of age. Three groups of three males and five females received infant formula containing nominal concentrations of 0, 300 and 1500 mg carrageenan/l. The carrageenan used was described as “food grade native carrageenan” with a molecular weight range of 197.1–394 kDa and was supplied to the baboons heated in the formula and bound to protein to stabilize the suspension of fat, protein and other nutrients. Baboons were fed formula from a freshly opened 120-ml disposable unit 5 times per day for the first 14 days, 4 times per day for the next 14 days, 3 times per day for the next 56 days and twice per day for 28 days until 112 days of age. No other food or fluid was provided. Formula intake was estimated as the difference between the weight of the bottle before and after feeding. Daily observations of the animals were made. Stools were tested weekly for occult blood. Body weight was measured daily, and organ weights at autopsy. Blood samples were taken monthly. On the night before autopsy, each baboon received water only, ad libitum. At autopsy under anaesthesia, the alimentary tract was removed from the rectum to the midpoint of the oesophagus. The intestines were opened, and the whole tract was pinned, serosa down, and fixed in 10% buffered formalin. The gut was examined minutely as fresh by at least two observers

and then again after fixation, often with a dissecting microscope. Fourteen blocks were taken from the alimentary tract for histological section. In addition, blocks from all major viscera, muscle, lymph nodes and the central nervous system were fixed in 10% formalin for microscopic analysis. Except for the amount of carrageenan, the formula composition was similar to most infant formulas on the market in the United States of America (USA) at the time of the study. Analysis of the formula revealed carrageenan concentrations of 255 ± 35 mg/l and 1220 ± 142 mg/l. The method used for analysis was modified from one used for infant formulas containing carrageenan in the range 200–300 mg/l. The authors stated that the validity of this modification was not known. Total formula consumption was reported in grams, therefore not enabling the equivalent carrageenan intake per baboon to be calculated (mg/kg bw per day), but the Joint FAO/WHO Expert Committee on Food Additives (JECFA) previously reported that the doses were equivalent to 86 and 432 mg/kg bw per day. Potential uptake of carrageenan into the circulation was not assessed.

Carrageenan in formula did not affect body weight, organ weights, urine and faecal characteristics or haematological and blood chemical parameters. No abnormal findings were noted on physical examination of the animals, nor were there any changes in the macroscopic or microscopic appearance of the gastrointestinal tract. Although a few abnormalities were noted in the alimentary tract for all dose groups, these were considered minor and unrelated to carrageenan treatment (McGill et al., 1977).

2.1.2 Special studies in vitro

A number of experiments were conducted in vitro in a human colonic epithelial cell line (NCM460), a primary rat ileal epithelial cell culture and fresh human ex vivo colonic tissue to determine the direct effects of carrageenan on intestinal cells. The intestinal epithelial cell line NCM460 and the rat ileal epithelial cells were treated with carrageenan (λ-, κ- and ι-carrageenan with an average molecular weight of 1000 kDa) at a concentration of 1 µg/ml for 1–96 h. Small colonic tissue fragments were obtained from colon specimens at the time of colectomy and treated with λ-carrageenan (with an average molecular weight of 1000 kDa at 1–10 µg/ml) for 1–24 h. Interleukin-8 (IL-8) secretion, IL-8 promoter activity, total and nuclear levels of nuclear factor (NF) κB, inhibitor protein IκBα, phosphorylated IκBα and Bcl10 protein were assessed by immunohistochemistry, western blot, enzyme-linked immunosorbent assay (ELISA) and complementary deoxyribonucleic acid (cDNA) microarray. Secretion of IL-8 significantly increased approximately 2-fold in all cells following λ-carrageenan exposure ($P < 0.001$). In the ex vivo colonic tissue, IL-8 secretion rose linearly in response to increasing concentrations of λ-, κ- or ι-carrageenan. The IL-8 response at 24 h following exposure to 1 µg/ml was greatest for λ-carrageenan (1273.0 ± 29.9 pg/ml per milligram protein), followed by κ-carrageenan (997.2 ± 41.7 pg/ml per milligram protein) and then ι-carrageenan (903.1 ± 41.4 pg/ml per milligram protein), compared with a baseline secretion of 624.6 ± 17.8 pg/ml per milligram protein.

There was a significant ($P < 0.001$) increase in total and nuclear levels of NFκB in NCM460 cells at 24 and 48 h following exposure to λ-carrageenan. At 24 h, the increases were 5-fold and 2-fold for total and nuclear NFκB, respectively. A significant ($P < 0.001$) increase in nuclear NFκB was also observed in the ileal epithelial cells following exposure to λ-carrageenan. Immunohistochemical staining for NFκB in the ex vivo colonic tissue demonstrated a marked increase in the intensity of staining. Tissue samples were exposed to 10 µg λ-carrageenan/ml for 2 h. NCM460 cells treated with λ-carrageenan for 24 and 48 h showed an increased phosphorylation of IκBα compared with controls. Exposure of NCM460 cells to λ-carrageenan for 96 h resulted in a significant ($P < 0.001$) increase in Bcl10, as demonstrated by cDNA microarray analysis. Increases in Bcl10 were also observed by western blot of the NCM460 whole cell lysate, following exposure to λ-, κ- and ι-carrageenan for 24 and 48 h. An increase in Bcl10 protein was observed by immunohistochemical staining in ex vivo colonic tissue samples treated for 2 h with 10 µg λ-carrageenan/ml. However, in the presence of 50 µmol caffeic acid phenethyl ester (an inhibitor of NFκB activation) per litre, there was a reduction in the up-regulation of IL-8 by carrageenan in NCM460 cells. The authors suggested that the carrageenan-induced increase in IL-8 secretion requires activated NFκB and is transcriptionally mediated. Bcl10 knockdown (by small interfering ribonucleic acid [RNA]) significantly ($P < 0.001$) reduced carrageenan-induced increases in IL-8 secretion in NCM460 cells. Also following knockdown of Bcl10 in NCM460 cells, there was a significant ($P < 0.001$) 50% decline in the carrageenan-induced increase of phosphorylated IκBα. The authors concluded that carrageenan stimulates an inflammatory cascade in normal colonic epithelial cells, involving Bcl10 activation, increase in phosphorylated IκBα, nuclear translocation of NFκB and increased production of IL-8 (Borthakur et al., 2007).

Primary cultures and subcultures of mammary myoepithelial cells from reduction mammoplasty samples were exposed to λ-carrageenan (1–14 µg/ml, molecular weight not stated) for 4–14 days and then investigated for ultrastructural changes. Cells were examined by electron microscopy, following staining for acid phosphatase and for aryl sulfatase. Carrageenan appeared to enter the cells by membrane-associated endocytic vesicles, which fused to form unusual petal-shaped arrays. Cells with the longest exposure (14 days) developed large endosomes and lysosomes with unusual linear inclusions. These inclusions arose from the membranes of the petal-like arrays and were associated with distortion and vacuolation of the lysosomes. In addition, the internal membrane traffic appeared to be disrupted by carrageenan exposure. Lysosomal distortion preceded destruction of the mammary myoepithelial cells, and the authors concluded that this association between exposure to carrageenan in tissue culture and destruction of mammary myoepithelial cells may be relevant to mammary malignancy in vivo (Tobacman & Walters, 2001).

In a similar experiment published only in abstract form, mammary myoepithelial cells in tissue culture were exposed to varying concentrations of λ-carrageenan (molecular weight not stated) for varying durations (not detailed) to determine the effects on steroid sulfatase in the cells. Enzyme activity of steroid sulfatase and messenger RNA (mRNA) expression were determined and compared

with measurements made for MCF-7 (a human breast adenocarcinoma cell line) cells. Steroid sulfatase enzyme activity was much greater in the mammary myoepithelial cells than in the MCF-7 cells. Following exposure to carrageenan, there was a concentration- and time-dependent decline in steroid sulfatase mRNA expression in the mammary myoepithelial cells. This decline in expression level, the authors suggested, was indicative of a feedback effect of carrageenan metabolism on sulfatase expression. The authors also suggested that down-regulation of steroid sulfatase may have significant effects throughout mammary tissue (Tobacman & Khalkhali-Ellis, 2002).

2.1.3 Special studies on tumour suppression and anti-tumour activity

A number of studies have utilized tumour models for evaluating degraded carrageenan fractions, with molecular weights in the range 1200–1726 Da. Haijin et al. (2003) and Yuan et al. (2006) determined the anti-tumour activity of κ-carrageenan oligosaccharides in the sarcoma 180 tumour mouse model. Liu et al. (2000) investigated the effect of ι-carrageenan on the adhesion of MCF-7 and MDA-MB231 adenocarcinoma breast cells to different substrata. Yuan & Song (2005) investigated the in vitro anti-tumour effects of κ-carrageenan in human neoplastic cell lines. Similarly, Zhou et al. (2004) tested samples of λ-carrageenan (molecular weights in the range 9.3–650 kDa) from *Chondrus ocellatus* in the S180 and H22 tumour models. Anti-tumour activity was significantly influenced by molecular weight ($P < 0.01$), with the two lowest molecular weight samples exhibiting the greatest inhibition of tumour growth (Zhou et al., 2004). These low molecular weight carrageenans were also shown to increase the anti-tumour activity of 5-fluorouracil (Zhou et al., 2005, 2006). The Committee concluded that these assays with low molecular weight carrageenans were not relevant to the oral exposure to food-grade carrageenan and therefore did not consider them further in the evaluation.

Using a cell culture–based human papillomavirus (HPV) inhibition assay, carrageenan has been identified as a potent infection inhibitor. Various types of carrageenan (λ-, κ- and ι-carrageenan, molecular weight not stated) exhibited inhibitory effects against the HPV 16 pseudovirus, with mean IC_{50} values ranging from 0.004 to 0.044 μg/ml. The addition of capsids increased the IC_{50} for carrageenan. In further flow cytometric analyses, the authors showed that ι-carrageenan blocked the binding of labelled capsids at various concentrations (concentrations not given). The authors concluded that carrageenan blocked papillomavirus infectivity in vitro primarily by preventing the binding of HPV virions to cells (Buck et al., 2006).

2.1.4 Special studies on the immune system

Inflammatory effects of degraded carrageenan (poligeenan) have been seen in studies in rodents, guinea-pigs and monkeys (Tobacman, 2001; Scientific Committee on Food, 2003). Other reviews have reported that there is no good evidence for adverse effects of undegraded, food-grade carrageenan (Cohen & Ito, 2002; Weiner et al., 2007).

There is some evidence that small amounts of (labelled) undegraded carrageenan may be able to cross the intestinal barrier, with accumulation of label in intestinal lymph nodes (Nicklin & Miller, 1989; Tobacman, 2001). A number of older studies have noted the uptake of carrageenan by intestinal macrophages, with subsequent migration of these macrophages to lymph nodes, spleen and liver (Grasso et al., 1973; Pittman et al., 1976; Oohashi et al., 1981; Nicklin & Miller, 1989; reviewed by Tobacman, 2001).

The effects of carrageenan on lymphocytes have been studied in various strains of mice (C57BL/6(B6), (BALB/c × B6)F_1, beige/beige, perforin(–/–), CD1d (–/–) and Vα14Jα281(–/–)). The various strains of mice were used to determine the differential effects of carrageenan on liver injury. λ-Carrageenan (molecular weight not stated) was purchased commercially and administered by a single intraperitoneal injection (1 mg/0.2 ml per mouse; equivalent to 50 mg/kg bw), and the mice were sacrificed at 3, 7 and 10 days. A number of phenotypic and functional characterizations were performed (preparations of hepatic mononuclear cells, immunofluorescence tests, cytotoxicity assays, depletion of natural killer [NK] cells and NK T cells,[1] histological analysis, assay of carrageenan binding, interferon-*gamma* [IFN-γ] secretion assay, reverse transcription-polymerase chain reaction [RT-PCR] for the detection of perforin). No death of phagocytic cells was detected. The proportion of NK cells increased in the liver, lungs and spleen and was accompanied by liver injury. The proportion of NK T cells increased only in the liver. A time-kinetic study in the liver showed activation of NK cells on days 3 and 7 and activation of NK T cells and extrathymic T cells on days 7 and 10. NK cell and NK T cell cytotoxicities were augmented in parallel with this activation.

Histological analysis of the liver revealed liver injury accompanied by hepatic necrosis. This was confirmed by the elevation of transaminases: glutamic oxaloacetic transaminase and glutamic pyruvic transaminase. Elimination of NK cells by the in vivo injection of anti-asialoGM_1 (α$AsGM_1$) antibody prior to the administration of carrageenen suppressed the liver injury. The authors thus concluded that NK cells were the major lymphocytes to induce the liver injury. The importance of perforin for NK cell–mediated liver injury was confirmed by using various strains of mice. The perforin(–/–) mice and beige/beige mice (which cannot secrete perforin) did not show liver injury. The NK T cell–deficient mice (CD1d(–/–) and Vα14Jα281(–/–)) showed severe liver injury. Perforin was also shown to gradually increase following the administration of carrageenan by the RT-PCR method. Based on these differential effects, the authors speculated that the major effector cells for the liver injury were NK cells and that the perforin molecules of NK cells are important in the induction of this injury.

Up to 40% of NK cells were shown to directly bind carrageenan, compared with less than 5% of NK T cells. This binding then induced a subsequent production of IFN-γ, with the major IFN-γ producer (up to 27%) being $CD3^-NK1^-1^+$ NK cells.

[1] NK cells are cytotoxic lymphocytes that play a role in the innate immune system, not requiring antigenic specificity; and NK T cells are lymphocytes that bridge the adaptive and innate immune systems, recognizing antigen presented by CD1d.

The authors concluded that primitive lymphocytes (mainly NK cells) might be important targets for the acute toxicity of carrageenan (Abe et al., 2002).

λ-Carrageenan (molecular weight not stated) was administered orally to female C3H/HeJ mice to determine its effect on sensitization to β-lactoglobulin. The mice were given drinking-water containing carrageenan (0.5 g/l; equivalent to 125 mg/kg bw per day, based on a default water consumption of 5 ml/day per mouse) and/or β-lactoglobulin (0.01 mg/l) ad libitum for 5 days before oral sensitization with β-lactoglobulin and cholera toxin. This treatment cycle was repeated 3 times. Two control groups received water containing either β-lactoglobulin (0.01 mg/l) or carrageenan (0.5 g/l) ad libitum for 5 days. The results were analysed by the Wilcoxon rank statistical test. After 28 days, mice were challenged with 10 mg β-lactoglobulin via gavage; anaphylactic reactions were scored, and the animals were sacrificed. Blood was collected for measuring antigen-specific antibodies.

The antigen challenge elicited reduced anaphylactic reactions in mice pretreated with the combination of carrageenan and β-lactoglobulin, compared with mice pretreated with carrageenan or β-lactoglobulin alone. Mesenteric lymph node cells and spleen cells from mice pretreated with carrageenan and β-lactoglobulin proliferated less in the presence of β-lactoglobulin or IL-2 than cells from control mice or mice receiving a single treatment. Production of β-lactoglobulin-specific antibodies was not suppressed by treatment with carrageenan and β-lactoglobulin. The authors concluded that administration of carrageenan in combination with an allergen (β-lactoglobulin), prior to sensitization, efficiently prevented anaphylaxis (Frossard et al., 2001).

In a similar study, the effect of carrageenan on allergic reactions was investigated. Experiments were performed on a range of female mice species (BALB/c, C3H/HeN, C57BL/6, C3H/HeJ, TLR2–/–, TLR4–/– and MyD88–/–). IFN-γ and IL-4 were measured in in vitro T cell–stimulated cultures. Cytokine production was measured as an indicator for immunity activation, and histamine release was induced by systemic injection of ovalbumin. Oral administration of λ-carrageenan (0.0002–0.02%; 2–200 µg/ml, molecular weight not stated) to immunized mice suppressed both antibody production and mitogen-induced T cell proliferation. Activation of immunity by carrageenan was found to be dependent on Toll-like receptor 4 (TLR4) and adaptor protein MyD88. Carrageenan was shown to enhance IFN-γ and reduce IL-4 in T cell cultures and decrease ovalbumin-specific immunoglobulin E (IgE) and inhibit histamine release upon systemic challenge. The authors concluded that λ-carrageenan would be a useful dietary supplement for the prevention and/or improvement of allergy-related diseases (Tsuji et al., 2003).

The Committee noted that although the above two studies suggest a beneficial effect of carrageenan on the immune system following oral administration, neither study provides specific details of the administered carrageenan. Immunostimulation via TLR4 suggests that the effects of carrageenan observed after oral administration do not necessarily require absorption into the systemic circulation.

A recent in vitro study indicates that λ-carrageenan (with an average molecular weight of 1000 kDa) induces inflammation in human intestinal epithelial cells in culture through a Bc110-mediated pathway that leads to NFκB and IL-8. Carrageenan may be immunogenic owing to its unusual α-1,3-galactosidic link, which is part of its disaccharide unit structure. This study suggests that carrageenan might have a role in intestinal inflammation and possibly inflammatory bowel disease, since Bc110 resembles NOD2 (the gene that activates NFκB), of which some mutations are associated with genetic susceptibility to Crohn disease (Borthakur et al., 2007).

2.2 Observations in humans

2.2.1 Clinical studies

In a study reviewed by the Committee at its fifty-first meeting, a sample of 1418 infants from the 1988 National Maternal and Infant Health Survey of the USA was examined to determine whether there was an association between the frequency of upper respiratory tract infection in the first 6 months of life and type of formula (liquid containing 0.03% carrageenan or powdered without carrageenan). A total of 1269 infants had consumed the liquid formula exclusively for the first 6 months of life, and 149 infants had consumed the powdered formula. The authors estimated that for those infants consuming the liquid formula, the average consumption of carrageenan over the first 6 months of life was 191 mg/day (equivalent to 38.2 mg/kg bw per day).

A slightly higher proportion of infants receiving liquid formula were free of upper respiratory tract infection for the first 6 months of life (53.4%) compared with those infants receiving powdered formula (47.6%) (statistical significance not provided). When the number of months of upper respiratory tract infection was analysed, the infants receiving liquid formula had an average of 0.9 months in which an upper respiratory tract infection occurred compared with 1.0 month for infants receiving powdered formula.

Using Poisson logistic-regression analysis, the risk of one or more colds in full-term infants during each month of the infants' first 6 months of life was associated with an odds ratio (OR) of 0.94 (95% confidence interval [CI] = 0.90–0.99, P = 0.015). Analysis of 364 preterm infants (333 of whom received liquid formula) showed no effect (OR = 1.05, 95% CI = 0.96–1.14, P = 0.277). The authors concluded that the data suggest that carrageenan-containing liquid infant formula is not immunosuppressive and not associated with an increased frequency of upper respiratory tract infections in either preterm or full-term infants (Sherry et al., 1993).

The Committee noted that this study did not address the possible effects of carrageenan on the gastrointestinal tract. The statistical power of the study is unlikely to have been sufficient to demonstrate an immunosuppressive effect of carrageenan.

In a randomized crossover study, carrageenan (type and molecular weight not stated) in powder form was incorporated into a yeast bread, a corn pudding, a gruel-like mixture of rice, chicken and spices and fish balls, prior to cooking by

standardized methods. These food items, which were four food items local to the Philippines, were given to 20 volunteers (4 males and 16 females, aged 28–61 years) to determine the effect of carrageenan on blood cholesterol and lipid levels. The dose of carrageenan was not reported, but the diet of each subject was arranged so as to provide approximately 40 g fibre/day. Subjects served as their own control, and each phase of the study (control and experimental) lasted for 8 weeks, separated by a 2-week washout. During the control phase, subjects consumed their regular diet; during the experimental phase, subjects were given the test foods to substitute part of their regular diet. During the experimental phase, each subject received daily two pieces of yeast bread (37.5 ± 2.5 g/piece) for breakfast and three pieces for snacks, as well as two 80-g portions of corn pudding for dessert. The chicken and rice dish (200 g) and fish balls (3 × 13 g) were given occasionally. Intakes of the experimental foods were monitored and recorded daily. Venous blood was extracted immediately before and after each phase for cholesterol and triglyceride analyses. Differences were determined by regression analysis.

Mean body weights did not differ significantly between the two groups. Mean intakes of iron, thiamine, riboflavin and fibre were all significantly higher during the experimental phase compared with the control phase. Subjects' mean serum cholesterol and serum triglyceride levels were significantly lower ($P < 0.0014$ and $P < 0.0006$, respectively) after 8 weeks of consumption of the test diet compared with the control phase. High-density lipoprotein (HDL) cholesterol was significantly higher ($P < 0.0071$) after 8 weeks on the experimental phase than after the control phase. Low-density lipoprotein (LDL) cholesterol, although lower after the experimental phase compared with the control phase, was not significantly different. The authors attributed the observed differences to the effect of a higher amount of fibre in the subjects' diet, with carrageenan being the main source of dietary fibre. Carrageenan was concluded to significantly reduce the blood cholesterol and lipid levels in the volunteers (Panlasigui et al., 2003).

The Committee noted that no other source of fibre was included in this study for comparison with carrageenan, but the authors referred to other studies testing other sources of fibre to draw comparisons. The decrease in serum cholesterol was higher in this study at 33% compared with other studies, where a 2–16% decrease in total cholesterol has been observed. The effect of fibre on serum triglyceride and HDL cholesterol levels is variable, with both increases and decreases being reported in other studies. Studies on fibre and lipid changes show that changes in LDL cholesterol are parallel to changes in serum cholesterol. However, in this respect, this study did not agree with the results of other studies. This study reported a non-significant decrease (6%) in LDL cholesterol, compared with other studies that report significant decreases of 5–20% in LDL cholesterol levels.

2.2.2 Post-marketing surveillance

Three International Formula Council member companies conducted an analysis of customer complaint records for their standard cow's milk–based or soy-based liquid formulas (both ready-to-feed and concentrate), which contain carrageenan; and the powder equivalents of these formulas, which do not contain

carrageenan. Complaints were compiled over a 56-month period (1 January 2002 – 31 August 2006) for two symptoms: blood in stool—because this is a symptom of colitis; and upper respiratory tract infections—to act as a control. In order to standardize the data from the different companies, a denominator of "total number of 8 fluid ounce servings distributed during the aforementioned period" was selected and calculated for each product.

The complaint rates for the control system (upper respiratory tract infections) were the same as blood in stool complaint rates. Overall, the complaint rates for blood in stool ranged from 0.10 to 0.54 per million servings. One carrageenan-containing product (ready-to-feed formula) showed higher complaint rates, whereas the other carrageenan-containing product (concentrate) showed a lower complaint rate than the powdered product (no carrageenan). Therefore, from these data, blood in stool was not specifically associated with the presence or absence of carrageenan in infant formula (Mountford, 2006).

The Committee noted that blood in stool is a very crude and often late sign of gastrointestinal damage (or cancer in adults). This analysis is based on records of customer complaints and therefore is not suitable for the safety assessment of carrageenan. More useful information to collect would be the objective assessment of occult blood in subgroups receiving formula with and without carrageenan.

2.2.3 Epidemiological studies

An epidemiological study was conducted to investigate the associations between the consumption of carrageenan and the incidence of mammary carcinoma (Tobacman et al., 2001, also reported as abstracts in Tobacman et al., 2000, 2002). A time-trend analysis with lag intervals of 10–35 years and Pearson and Spearman correlation coefficients was undertaken to determine if carrageenan intake (along with the intake of 12 other water-soluble polymers) had a positive or negative correlation over time with the incidence of mammary carcinoma in the USA. Incidence data for breast cancer and carrageenan intake data were obtained from published sources. Eighty per cent of the total consumption of carrageenan was considered as food consumption, with the remainder attributed to products such as toothpaste, deodorants and room deodorizers. Statistically significant correlations were found for Pearson correlation at 25 and 30 years ($r = 0.88$, $P = 0.048$; $r = 0.96$, $P = 0.042$, respectively); and Spearman correlation at 30 years ($r = 1.000$, $P < 0.0001$). The authors concluded that increased consumption of several gums (including carrageenan) correlates positively with an increased incidence of breast cancer (Tobacman et al., 2001). The Scientific Committee on Food (2003) concluded that these data did not support a causal relationship between breast cancer incidence and intakes of carrageenan.

The Committee also concluded that this comparison of gross carrageenan consumption in the USA with national breast cancer incidence rates cannot prove causation. No adjustment was made for non-food consumption, and consumption was analysed as though it was distributed equally throughout the population. Similarly, no adjustments were made for possible relationships between the 13 gums studied and any of the acknowledged risk factors for mammary carcinoma.

2.2.4 Case reports

In discussions by an International Expert Group reviewing the requirements for a global infant formula standard, some concern was raised regarding possible greater sensitivity of infants to gastrointestinal effects of carrageenan compared with adolescents or adults. The International Expert Group concluded that "given the lack of adequate information on possible absorption of carrageenan by the immature gut in the young infants and its biologic effects in infancy, it appears inadvisable to use carrageenan in infant formulae intended for feeding young infants, including those in the category of foods for special medical purposes" (Koletzko et al., 2005).

The concern expressed by the International Expert Group appears to be based on a report of a reaction to a barium enema in which a 26-year-old suffered from anaphylaxis due to carrageenan (0.45% weight per volume sodium carrageenan) (Tarlo et al., 1995). The authors confirmed this by later skin prick tests and testing the patient's serum for IgE antibodies to carrageenan. The carrageenan supplied for the manufacture of the barium enema came from a company that uses blends of 10 different types of carrageenans; the carrageenan used for the skin test was not detailed. A trade association that represents worldwide producers of seaweed extracts suggested that the barium enema was very likely composed of poligeenan rather than carrageenan, as poligeenan is the primary current excipient in barium enemas for the upper gastrointestinal tract (Kirsch, 2006).

3. DIETARY EXPOSURE

3.1 Exposure to undegraded carrageenan

The Committee previously estimated that exposure to carrageenan or processed *Eucheuma* seaweed from their use as food additives would be in the region of 30–50 mg/person per day (Annex 1, reference *154*). No new data were submitted relating to exposure of the general population to carrageenan.

The focus of the current assessment is use of carrageenans in infant liquid formulas. It has been proposed that carrageenan could be used at maximum concentrations of 0.03 g/100 ml in milk- and soy-based formulas and 0.1 g/100 ml in hydrolysed protein– and/or amino acid–based liquid infant formulas.

The consumption of formula by infants can be calculated based on 125 kcal/kg bw per day and on a content of 0.8 kcal/g in formula. Therefore, the resulting dietary exposure (E) from formulas can be estimated for concentrations of 0.03% and 0.1%, respectively, as:

$E = 125/0.8 \times 0.0003 = 0.047$ g/kg bw per day
$E = 125/0.8 \times 0.001 = 0.16$ g/kg bw per day

Based on the World Health Organization (WHO) Child Growth Standards, the median body weights for boys at 3, 6 and 12 months of age are, respectively, 6.4, 7.9 and 9.6 kg, and the high percentiles (+2 SD) are, respectively, 8.0, 9.8 and 12 kg.

Table 1. Estimates of dietary exposure of infants aged 3–12 months to carrageenan from infant formula

Age (months)	Body weight (kg)	% formula	Exposure (g/day) at 0.03%[a]	Exposure (g/day) at 0.1%[a]
3 (median)	6.4	91.1	0.27	0.91
3 (+2 SD)	8.0	91.1	0.34	1.14
6 (median)	7.9	43.3	0.16	0.53
6 (+2 SD)	9.8	43.3	0.20	0.66
12 (median)	9.6	13.7	0.06	0.21
12 (+2 SD)	12	13.7	0.08	0.26

[a] Assuming consumption of 125 kcal/kg bw per day and a content of 0.8 kcal/g in formula.

The consumption of formula was reported by Boggio et al. (1999) in France to be 91.1%, 43.3% and 13.7% of the total caloric intake at, respectively, 3, 6 and 12 months of age. Estimates of dietary exposure to carrageenan from infant formulas are shown in Table 1.

These figures are confirmed by results from surveys on the consumption of liquid formulas by infants. Koletzko et al. (2000) reported from Germany a maximum average consumption of 728.8 ± 148.2 ml/day at 6 months of age. This level of consumption is similar to the mean level of 750 ml/day reported in France by Boggio et al. (1999) for infants 3 months old. If the reported average consumption plus 2 standard deviations is considered, to take into account interindividual variability, this would result in a daily consumption of 1025 ml (729 + (2 × 148)).

More generally, these figures are also consistent with the recommended consumption per meal ranging from 150 to 240 ml at, respectively, 3 and 6 months of age, and the number of meals is between four and six. An assumption of four meals of 240 ml would result in a total consumption of 960 ml/day.

On the basis of a mean consumption of 750 ml and a high consumption of 1025 ml, the daily exposure to carrageenan from liquid infant formulas can be estimated to be between 0.20 g/day (0.03 × 750/100) and 0.30 g/day (0.03 × 1025/100) for milk- and soy-based formulas. For hydrolysed protein– and/or amino acid–based liquid infant formulas, the daily exposure to carrageenan can be estimated to be between 0.75 g/day (0.1 × 750/100) and 1.0 g/day (0.1 × 1025/100).

3.2 Exposure to degraded carrageenan

Tobacman (2001, 2003) discussed the possibilities of contamination of carrageenan by low molecular weight components and of degradation during food processing, noting that in a study reported in 1983, 25% of carrageenans in eight food-grade carrageenans were found to have a molecular weight below 100 kDa. The sponsor stated that such contamination accidents would be detected through quality control (QC) procedures; in addition, carrageenan contaminated with low

molecular weight material would not pass routine QC specifications (Marinalg International, 2006).

Tobacman (2003) also noted that heat exposure (up to 75 ºC) of foods combined with low pH (<6) can lead to the degradation of food-grade carrageenan during food processing. Cohen & Ito (2002) reported that carrageenan is extracted from seaweeds under alkaline conditions, and degradation is avoided. The sponsor has concluded that food processing protocols ensure that minimal degradation of carrageenan will occur (Marinalg International, 2006); once carrageenan is in the gel configuration, it becomes highly resistant to degradation (Cohen & Ito, 2006).

No information was available to the Committee regarding exposure to degraded carrageenan.

4. COMMENTS

4.1 Toxicological data

There was a lack of clarity regarding the types of carrageenan used in some published studies and whether they relate to food-grade carrageenan, which has an average molecular weight well above 100 kDa, or to poligeenan, which has an average molecular weight of 20–30 kDa and is also known as degraded carrageenan. Poligeenan has been widely used as an inflammatory and adjuvant agent in experimental models for investigation of immune processes. Very little is known about the possible degradation of food-grade carrageenan to lower molecular weight species and possible subsequent effects, although some evidence indicates that gastrointestinal metabolism of carrageenan by acid digestion and bacterial degradation to lower molecular weight components might occur. At its fifty-first meeting, the Committee concluded that such breakdown is probably of limited toxicological significance since, if native carrageenan were sufficiently degraded to cause ulceration or tumour growth, this would be detected in feeding studies. Some bacteria are known to hydrolyse carrageenan, resulting in low molecular weight forms. These bacteria, however, are of marine origin, and it is not known if the human microbial flora can perform similar hydrolysis reactions.

Intestinal absorption studies are lacking both for humans and for animals, in vitro and in vivo. At its thirteenth meeting, the Committee commented that little carrageenan is absorbed when ingested by several animal species; subsequently, at its seventeenth meeting, the Committee commented that high molecular weight carrageenan is probably not absorbed. The European Commission's Scientific Committee on Food more recently concluded that it could not be excluded that carrageenan might be absorbed by the immature gut and that absorbed material might affect the immune system in the infant. No new data have been published addressing this issue.

Of the information reviewed previously by the Committee, all animal experiments apart from one in baboons were performed in adult animals and not in suckling infants, which limits their usefulness for the safety evaluation of carrageenan for infants. From the absence of effects in the study in infant baboons

fed infant formula containing carrageenan, a no-observed-effect level (NOEL) of 1220 mg/l in formula was identified, equivalent to 432 mg/kg bw per day. However, in this study, the colon was fixed in 10% buffered formalin, and this does not enable identification of mast cells that would be present if an inflammatory process had been initiated. Mast cells of the gastrointestinal tract mucosa can be visualized only using special fixation techniques, because they differ from other mast cells with respect to the spatial arrangement of glycosaminoglycan and protein in their granules.

One new study conducted in mice showed that carrageenan enhanced the tumorigenicity of a carcinogen, MNU, confirming the results of studies previously evaluated by the Committee at its fifty-seventh meeting.

The Committee noted at its fifty-first and fifty-seventh meetings that proliferative effects on the gastrointestinal tract were reported in a number of studies of rats fed dietary concentrations of 2.6% carrageenan, equivalent to 1300 mg/kg bw per day, or greater. No effects were observed in rats at carrageenan concentrations of up to 1.5% in the diet, equivalent to 750 mg/kg bw per day. Proliferative and inflammatory effects were observed in one new study in mice administered λ-carrageenan in the drinking-water at concentrations of 1% and 4% (equivalent to approximately 1100 and 3500 mg/kg bw per day, respectively). A NOAEL was not identified in the mouse study, but the lowest-observed-adverse-effect level (LOAEL), expressed on a body weight basis, was similar to that in the rat.

Effects on the gastrointestinal tract were not reported in a recent 90-day dietary study in rats with food-grade κ-carrageenan with a low molecular weight tail fraction (7% below 50 kDa). No effects were observed at the highest dietary concentration of 50 000 mg/kg, which was equivalent to 3394 mg/kg bw per day. As with the baboon study referred to above, this study would not have identified mast cells.

A number of in vitro mechanistic studies were available. These were of limited value for the safety assessment of dietary carrageenan. Both activation and suppression of the immune system have been reported, apparently involving both nonspecific (macrophage) and specific (lymphocyte) immune responses. Studies evaluating the possible effects on the immune system were limited, in that they used either systemically administered carrageenan or lower molecular weight forms of carrageenan. There was little information with respect to possible effects on the immune system in humans following oral exposure to food-grade carrageenan.

Presumed mechanisms of intestinal injury following oral administration of carrageenan in rodents include damage via free oxygen radicals. Activation of thymidine kinase in epithelial cells of the gut as a sign of increased cell turnover has also been considered. It is still unclear whether the low absorption of carrageenan via the intestinal tract could affect the immunity of the host. It is also unclear whether absorption may be greater in the neonate, during weaning and in adults and children following allergic reactions and episodes of gastrointestinal disease.

No reports have been identified that address the particular question of effects on the immature intestine and immunity in experimental models or in prospectively designed human studies.

Data submitted summarizing customer complaint records for cow's milk– and soy-based infant formulas with and without carrageenan content did not reveal statistical differences between these groups with respect to blood in stool or upper respiratory tract infections. The Committee noted that these records did not relate to hydrolysed protein– and/or amino acid–based liquid formulas and that such reports would be unlikely to reveal subtle adverse effects. One epidemiological study indicated an association between consumption of carrageenan and incidence of mammary cancer. The Committee concluded that these data did not support a causal relationship because of limitations in the methodology and lack of adjustments for acknowledged risk factors for mammary carcinoma.

4.2 Assessment of dietary exposure

The draft Codex infant formula standard (Codex Alimentarius Commission, 2006b), in section 4.1.7, proposes the following maximum levels in the product ready for consumption:

- 0.03 g/100 ml for regular milk- and soy-based liquid formulas;
- 0.1 g/100 ml for hydrolysed protein– and/or amino acid–based liquid formulas.

The consumption of formula by infants can be calculated based on the caloric requirement of 125 kcal/kg bw per day and on a content of 0.8 kcal/g in formula in order to provide a realistic estimate of dietary exposure to carrageenan.

The average daily exposure to carrageenan from liquid infant formulas was estimated to be 47 mg/kg bw per day for milk- and soy-based formulas (0.03% carrageenan) and 160 mg/kg bw per day for hydrolysed protein– and/or amino acid–based liquid infant formulas (0.1% carrageenan). These exposure estimates apply to infants fed exclusively on formula.

The Committee also estimated exposure to carrageenan of infants of 12 months of age, based on a survey in France showing that consumption of formula represents 13.7% of total caloric intake at this age. Mean exposures were 6 mg/kg bw per day for milk- and soy-based formulas (0.03% carrageenan) and 22 mg/kg bw per day for hydrolysed protein– and/or amino acid–based liquid infant formulas (0.1% carrageenan).

5. EVALUATION

As a general principle, the Committee considers that the ADI is not applicable to infants under the age of 12 weeks, in the absence of specific data to demonstrate safety for this age group.

No studies were available addressing effects of carrageenan on the immature gut, and it was not possible to draw conclusions on whether carrageenan might be absorbed by the immature gut. In addition, there were limited data to

indicate whether or not carrageenan can affect the immune response of the gastrointestinal tract, and the nature and potential consequences of such an effect are unknown.

Potential effects of carrageenan in infants could arise from a direct action on the epithelium of the intestinal tract, which would be related to the concentration of carrageenan in infant formula. Alternatively, potential effects could arise from absorption of the low molecular weight fraction of carrageenan, which would be more likely to be related to the dietary exposure expressed on a body weight basis. Therefore, the Committee considered both the concentration of and exposure to carrageenan. The margin of exposure between the concentration in drinking-water reported to cause inflammatory effects in mice and the maximum concentration (0.1%) of carrageenan used in infant formula was 10. On a body weight basis, at this maximum concentration, there was a margin of exposure of about 7 between the lowest doses reported to cause inflammatory responses in rats and mice (1100–1300 mg/kg bw per day) and the estimated exposure to carrageenan from infant formula of 160 mg/kg bw per day prior to weaning. For infants of 12 months of age, there was a margin of exposure of 50 between the lowest-effect doses in rats and mice and the estimated mean exposure to 0.1% carrageenan in infant formula and a margin of exposure of 180 for 0.03% carrageenan, not taking into account possible exposure to carrageenan from other foods. The Committee considered all of these margins of exposure to be insufficient to ensure protection of infants fed infant formula containing carrageenan. The Committee was therefore of the view that it is inadvisable to use carrageenan or processed *Eucheuma* seaweed in infant formula intended for infants up to and including 12 months of age.

The Committee previously concluded that the NOEL of 750 mg/kg bw per day for inflammatory responses in the gastrointestinal tract greatly exceeded the estimated human intake of carrageenan or processed *Eucheuma* seaweed of 30–50 mg/person per day from their use as food additives and therefore allocated a group ADI “not specified”. The new information available to the Committee did not alter this conclusion. The group ADI “not specified” for the sum of carrageenan and processed *Eucheuma* seaweed was maintained for food additive uses in foods other than infant formula.

5.1 Recommendation

The Committee noted that the previous dietary exposure estimate for carrageenan was made solely using production poundage and may be outdated. The Committee therefore recommended that a new dietary exposure evaluation, employing specific food type and use level information, be undertaken, ensuring that new uses are adequately taken into consideration.

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CYCLOTETRAGLUCOSE AND CYCLOTETRAGLUCOSE SYRUP

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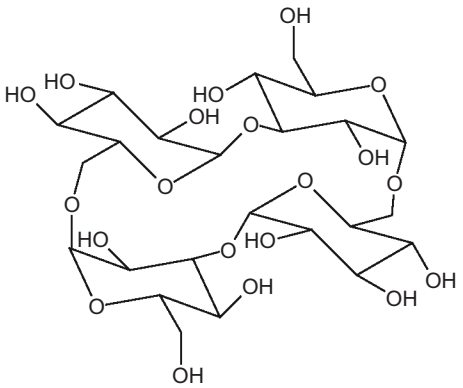
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1. EXPLANATION

Cyclotetraglucose (Figure 1) was placed on the agenda at the request of the thirty-eighth meeting of the Codex Committee on Food Additives and Contaminants under the name cyclotetraose (Codex Alimentarius Commission, 2006). The Committee considered that the name cyclotetraose was misleading, as it suggests that the substance is a four-carbon sugar, whereas it is actually a cyclic tetramer of glucose. The Committee therefore assigned it the name cyclotetraglucose. In reaching its decision, the Committee took into account the principles on nomenclature elaborated at its thirty-third meeting (Annex 1, reference *83*). The Committee received information on two types of products, cyclotetraglucose and cyclotetraglucose syrup.

Figure 1. Chemical structure of cyclotetraglucose



Cyclotetraglucose occurs naturally in sake lees (i.e. the sediment that forms during rice wine production), in sake itself and in the cells of *Saccharomyces cerevisiae*. Cyclotetraglucose is a non-reducing cyclic tetrasaccharide consisting of four D-glucopyranosyl units linked by alternating α(1→3) and α(1→6) glycosidic bonds. The chemical name is *cyclo*[→6)-α-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→3)-α-D-glucopyranosyl-(1→].

Cyclotetraglucose is produced from hydrolysed food-grade starch by the action of a mixture of 6-α-glucosyltransferase (6-GT) and α-isomaltosyltransferase (IMT) derived from *Sporosarcina globispora* and cyclodextrin glycosyltransferase (CGTase) derived from *Bacillus stearothermophilus*. After purification, the product is obtained as either cyclotetraglucose or cyclotetraglucose syrup. Cyclotetraglucose contains not less than 98% cyclotetraglucose, whereas cyclotetraglucose syrup contains 30–40% cyclotetraglucose, both calculated on the anhydrous basis. Cyclotetraglucose and its branched derivatives comprise about 45–55% of cyclotetraglucose syrup. The syrup also contains 15–20% mono-, di- and trisaccharides, as well as about 30% of a variety of unidentified saccharides.

Cyclotetraglucose was placed on the agenda for evaluation as a carrier and stabilizer; however, the manufacturer indicated that cyclotetraglucose and cyclotetraglucose syrup could be used as a dietary fibre. The Committee evaluated cyclotetraglucose for use in food as a carrier for flavours, polyunsaturated fatty acids and vitamins and as a food ingredient. It is stressed that the Committee evaluated the safety of the estimated dietary exposures to cyclotetraglucose resulting from the proposed use levels as a food ingredient only, assuming that these encompassed the much lower levels of use as a carrier and stabilizer. At its sixty-third meeting, the Committee noted that the evaluation of health, nutrient or other claims for food ingredients is outside its remit (Annex 1, reference *173*). Therefore, the Committee did not assess the merit of cyclotetraglucose or cyclotetraglucose syrup as a dietary fibre.

Cyclotetraglucose and cyclotetraglucose syrup have not been previously evaluated by the Committee.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Digestibility in vitro

In vitro experiments have shown that cyclotetraglucose dissolved in Bis-Tris buffer (50 mmol/l) is not degraded by human salivary or porcine pancreatic α-amylase or by artificial gastric juice (pH 2). Only 0.7% of cyclotetraglucose incurred ring opening during a 3-h incubation period with an acetone powder preparation of the rat intestinal mucosa to form a linear tetrasaccharide (Hashimoto et al., 2006).

2.1.2 Digestibility in vivo

(a) Rats

The non-digestibility of cyclotetraglucose was demonstrated in a study conducted with a group of 35 non-adapted male Wistar rats administered a single oral gavage dose of 100 mg cyclotetraglucose/kg body weight (bw) (provided as a 10% cyclotetraglucose solution, solvent not reported). Blood samples were collected from the portal vein before dosing and at 2, 4, 6, 12, 24 and 48 h post-dose administration (seven rats per time point). The rats were killed at each blood collection time point, and the cyclotetraglucose content of several organs (i.e. stomach, duodenum, jejunum, ileum, caecum and colon) was determined. Approximately 94% of the administered cyclotetraglucose dose was recovered in the faeces, and the remaining portion (6%) was detected in the gastrointestinal tract at 12 h post-dose administration. No cyclotetraglucose was detected in the blood during the experimental period (Hashimoto et al., 2006).

(b) Humans

Indirect evidence for the non-digestibility of cyclotetraglucose in humans is provided by a study in which a group of 18 healthy volunteers (12 males and 6 females) ingested 30 g of either glucose (control) or cyclotetraglucose dissolved in 120 ml of water after an overnight fast. Blood cyclotetraglucose, glucose and insulin levels were measured at regular intervals for up to 2 h after dosing. The lack of a glycaemic and insulinaemic response after cyclotetraglucose intake and the absence of detectable levels of cyclotetraglucose in blood samples (using gas chromatography) demonstrated that this cyclic oligosaccharide is not hydrolysed to glucose in the human small intestine (Miwa et al., 2005a).

Cyclotetraglucose syrup contains, in addition to cyclotetraglucose and branched cyclotetraglucose derivatives, nearly 30% (dry basis) other linear oligosaccharides resulting from the enzymatic degradation of starch during the production process. Six healthy males and three healthy females were each provided 50 g of dry solids from cyclotetraglucose syrup dissolved in 200 ml of water or glucose (control) following an overnight fast. Blood samples were obtained at regular intervals after dosing to assess blood cyclotetraglucose, glucose and insulin levels. Peak blood glucose levels, time to attain the maximum concentration and the area under the curve (AUC) for blood glucose following cyclotetraglucose syrup solids ingestion were comparable to those following glucose consumption; however, serum insulin levels and the AUC for serum insulin were significantly lower following cyclotetraglucose syrup solids ingestion than after glucose consumption. Non-glucose saccharides were detected in the blood of six subjects consuming the cyclotetraglucose syrup solids. Since consumption of cyclotetraglucose syrup solids, but not cyclotetraglucose (Miwa et al., 2005a), was associated with a significant glycaemic response, it can be inferred that the other oligosaccharide constituents of the syrup are readily digested and absorbed (Miwa et al., 2005b).

2.1.3 Fermentation in vitro

The results of an in vitro fermentation experiment with the caecal contents of non-adapted male Wistar rats demonstrated that a solution of cyclotetraglucose (solvent not reported) is only slowly degraded by the intestinal microflora (about 7% within 12 h) (Hashimoto et al., 2006). In another in vitro fermentation assay in which the utilization of cyclotetraglucose (dissolved in a peptone yeast extract solution) by 22 strains of human intestinal bacteria was compared with that of glucose by measuring the pH of each inoculated medium following a 96-h incubation period (a pH of $\geq$6.0 was reflective of no utilization of test material), none of the bacteria were able to degrade cyclotetraglucose (Hashimoto et al., 2006).

However, when fresh stool samples obtained from 10 healthy male human subjects were incubated with cyclotetraglucose dissolved in 0.1 mol sodium bicarbonate/l solution for a period of 24 h, approximately <5–25% of the cyclotetraglucose test material was degraded in the presence of the faecal samples obtained from 9 of the 10 subjects within the first 6 h. In the remaining subject, degradation of cyclotetraglucose proceeded at a faster rate, reaching about 70% at the end of the 6-h period. At the end of the 24-h incubation period, more than 90%

of the cyclotetraglucose was fermented in the presence of stool samples from five subjects, including complete fermentation in one subject. Conversely, faecal samples of two subjects fermented less than 5% of the cyclotetraglucose, whereas in the remaining three stool samples, fermentation at 24 h varied from approximately 30% to 65% (Oku, 2005).

A similarly variable fermentation profile was observed when human faecal samples were incubated with cyclotetraglucose syrup (Oku, 2005).

2.2 Toxicological studies

2.2.1 Acute toxicity

The results of studies of the acute toxicity of cyclotetraglucose and cyclotetraglucose syrup in rats treated dermally or orally are shown in Table 1.

(a) Cyclotetraglucose

Groups of five male and five female 8- to 10-week-old Crl:CD (SD)IGS BR rats were administered single doses of cyclotetraglucose dissolved in purified water at dose levels of 200, 2000 or 5000 mg/kg bw by oral gavage after a 17- to 20-h fast. A control group was not included. The rats were observed immediately after and at approximately 1, 2.5 and 4 h post–dose administration and daily thereafter for a period of 15 days for clinical signs of toxicity and mortality. Body weights were recorded on days 0 (day of dose administration), 7, 14 and 15. All animals were killed on day 15 and subjected to gross necropsy. No mortality or signs of toxicity were noted in response to the treatment with cyclotetraglucose. Body weights and weight gains did not differ between the groups. Gross necropsy did not reveal any visible abnormalities (Vegarra, 2001).

(b) Cyclotetraglucose syrup

Wistar albino rats (five per sex) were administered a single dose of 5000 mg cyclotetraglucose syrup/kg bw by oral gavage. No control group was indicated. Since the total solids comprised 72.0% of the syrup's composition, of which cyclotetraglucose accounted for 36.4%, a 5000 mg/kg bw dose of cyclotetraglucose syrup provided approximately 1310 mg cyclotetraglucose/kg bw. The rats were observed for clinical signs and symptoms of toxicity and mortality at 1, 2 and 4 h post–dose administration and once daily thereafter for a period of 14 days. At the

Table 1. Acute toxicity of cyclotetraglucose and cyclotetraglucose syrup

Species	Sex	Route	LD_{50} (mg/kg bw)	Reference
Cyclotetraglucose				
Rat	Male and female	Oral	>5000	Vegarra (2001)
Cyclotetraglucose syrup				
Rat	Male and female	Oral	>5000	Cerven (2004a)

end of the observation period, all animals were killed and subjected to a gross pathological examination. None of the animals died during the study period, and all animals gained weight; body weight changes were reported to be "normal". A single cyclotetraglucose-treated female exhibited localized alopecia (on the front paws), which developed on day 11 and persisted until the end of the observation period, whereas chromodacryorrhoea was noted in one male in the test group on the last day of the study period (day 14). No systemic or pathological abnormalities were observed in any of the other animals (Cerven, 2004a).

2.2.2 Short-term studies of toxicity

All of the short-term studies reviewed below were conducted on rats.

(a) Cyclotetraglucose

In a 90-day oral toxicity study designed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (1998), groups of 10 male and 10 female Wistar albino rats received diets (Purina Certified Rodent Chow) with 0%, 2.5%, 5% or 10% cyclotetraglucose (pentahydrate). Based on the measured weekly food intakes, dietary administration of 2.5%, 5% or 10% cyclotetraglucose resulted in mean daily dose levels of 1568, 3012 and 6333 mg cyclotetraglucose/kg bw for male rats and 1799, 3597 and 7270 mg cyclotetraglucose/kg bw for female rats, respectively. The homogeneity, stability and concentration of cyclotetraglucose in the test diets were confirmed by high-performance liquid chromatographic (HPLC) analyses. On day 1 of the study, the animals were about 7 weeks old. They were housed individually in wire mesh cages with paper bedding. Drinking-water and test diets were provided ad libitum. Food intakes were determined on a weekly basis throughout the study period. Body weights were recorded at the start and the end of the study and also at weekly intervals throughout the study period. Ophthalmoscopic examinations were performed prior to the start of the study and within 1 week of termination. During the last 2 weeks of the study, a functional observational battery (FOB) was conducted on each animal at every dose level. All animals were fasted overnight on day 90 and, on the following day, anaesthetized with ether and exsanguinated by collecting blood from the dorsal aorta. Blood samples were analysed for standard haematological and clinical chemistry parameters. The weights of the liver, brain, adrenals, kidneys, spleen, testes and epididymides (males only), ovaries and uterus (females only), thymus and heart were recorded. Complete histopathological examination of all major organs and tissues obtained from all control and high-dose animals was conducted.

All animals survived until the end of the study. Soiling of the anogenital area and transient laxation were noted in a few animals of the mid- and high-dose groups. Several of the control and test female rats exhibited alopecia of the forelimbs. Body weights did not differ significantly between treated groups and the control group at any time during the study. Food consumption also was not affected by treatment with cyclotetraglucose. The FOB did not reveal any significant, treatment-related differences between the control and test groups. Except for a statistically significant, but slight, decrease in the mean corpuscular haemoglobin

concentration in mid-dose males, there were no other differences in haematological parameters between treated groups and controls. The clinico-chemical analyses revealed statistically significant variations in calcium (increased in all groups of treated females and low-dose males), triglyceride (decreased in low-dose males and females) and phosphorus (increased in high-dose males) levels between test and control animals; however, the variations were not dose related (i.e. calcium and triglycerides) and did not occur consistently in both sexes of animals (phosphorus). Given the sporadic nature of the haematological and clinico-chemical variations and the absence of any accompanying histopathological changes, the differences were not considered to be biologically significant. No treatment-related differences were observed in absolute or relative organ weights between control and test animals. The ophthalmoscopic examination revealed no sign of ocular toxicity related to treatment with cyclotetraglucose.

Both gross necropsy and histopathology revealed only isolated incidences of sporadic variations, consisting of focal chronic inflammation of the liver, heart, kidneys, lungs, prostate and trachea, multifocal chronic nephropathy in the kidneys, dilated mucosal glands in the stomach, hyperplasia of the cervical lymph nodes, and pigmented macrophages, multifocal necrosis, vacuolation and hepatodiaphragmatic nodules in the liver, which occurred in both controls and test animals and are typically encountered in rats of this age and strain. Thus, none of the variations were considered to be related to treatment with dietary cyclotetraglucose. Based on the results of this study, it was concluded that the ingestion of cyclotetraglucose for a period of 90 days at dietary levels of up to 10% was well tolerated and did not produce any adverse effects in rats. Accordingly, the highest tested dietary cyclotetraglucose concentration of 10%, corresponding to intakes of 6333 and 7270 mg cyclotetraglucose/kg bw per day for male and female rats, respectively, was established as the no-observed-effect level (NOEL) (Cerven, 2004b).

(b) Cyclotetraglucose syrup

In a 91-day oral toxicity study, which was designed according to OECD Test Guideline 408 (1998), groups of 10 male and 10 female Wistar albino rats received diets (Purina Certified Rodent Chow) with 0%, 2.5%, 5% or 10% added cyclotetraglucose syrup. The total solids content of the test material was approximately 72%, with 36–37% of the dry matter accounted for by cyclotetraglucose. Based on the measured weekly food intakes, dietary administration of 2.5%, 5% or 10% cyclotetraglucose syrup resulted in mean daily dose levels of 1573, 3165 and 6687 mg cyclotetraglucose syrup/kg bw for male rats and 1738, 3641 and 7177 mg cyclotetraglucose syrup/kg bw for female rats, respectively. Since cyclotetraglucose comprised approximately 26% of the syrup, male rats ingested daily 409, 823 and 1739 cyclotetraglucose/kg bw, whereas females were exposed to daily dose levels of 452, 947 and 1867 cyclotetraglucose/kg bw at the low-, mid- and high-dose levels, respectively. The homogeneity, stability and concentration of cyclotetraglucose, the main component of the cyclotetraglucose syrup, in the test diets were confirmed by HPLC analyses. On day 1 of the study period, the animals were about 7 weeks old. They were housed

individually in wire mesh cages with paper bedding. Drinking-water and test diets were provided ad libitum. Throughout the study, the animals were observed daily for mortality and clinical signs of toxicity. Food intakes were determined on a weekly basis throughout the study period. Body weights were recorded at the start and end of the study and also at weekly intervals throughout the study period. Ophthalmoscopic examinations were performed prior to study initiation and 1 day before termination. During the last 2 weeks of the study, a FOB was conducted on each animal at every dose level. All animals were fasted overnight on day 91 and, on the following day, were anaesthetized with ether and exsanguinated by collecting blood from the dorsal aorta. Blood samples were analysed for standard haematological and clinical chemistry parameters. The weights of the liver, brain, adrenals, kidneys, spleen, testes and epididymides (males only), ovaries and uterus (females only), thymus and heart were recorded. Complete histopathological examination of all major organs and tissues was conducted in all animals of the control and high-dose groups.

All animals survived until the end of the study. Laxative effects were not observed in any of the groups, and soiling of the anogenital area was noted in only a single male animal of the high-dose group on day 3. With the exception of a significantly higher food intake in females of the high-dose group in week 5, which was considered to be fortuitous, food consumption of all test groups was comparable to that of controls. Body weights did not differ significantly between treated groups and the control group at any time during the study. The daily observations for clinical signs or symptoms of toxicity were reported to be sporadic and non–dose dependent and occurred with similar frequency in control and test animals. The FOB did not reveal any significant treatment-related differences between test and control animals. There were no differences in any of the evaluated haematological parameters between treated groups and controls. In comparison with the controls, the clinico-chemical analyses revealed only significant increases in mean levels of glucose and total bilirubin in males of the low-dose group. In light of the absence of a dose-related response and any relevant histopathological changes, the changes in clinico-chemistry values were considered to lack biological significance. Neither absolute nor relative organ weights were affected by the dietary treatment with cyclotetraglucose syrup. The ophthalmoscopic examination also revealed no signs of ocular toxicity attributable to the treatment.

Abnormalities observed at gross necropsy were limited to incidental occurrences of non-dose-related changes. Likewise, with the exception of changes that are typically encountered in rats of this age and strain and that were considered to be not related to the dietary administration of cyclotetraglucose (including multifocal chronic nephropathy in the kidneys, mucosal glands in the stomach, focal or multifocal chronic inflammation of the liver, heart and prostate, hyperplasia of the cervical lymph nodes and vacuolation in the liver), the histopathological examination of organs and tissues of the control and high-dose group was unremarkable. It was therefore concluded that the ingestion of cyclotetraglucose syrup for a period of 91 days at dietary levels of up to 10% was well tolerated and did not produce any adverse effects in rats. Under the conditions of this study, the highest tested dietary concentration of 10%, equivalent to 6687 and 7177 mg cyclotetraglucose syrup/kg

bw per day for male and female rats, respectively (1739 and 1867 mg cyclotetraglucose in males and females, respectively), was determined to be the NOEL (Cerven, 2004c).

2.2.3 *Long-term studies of toxicity and carcinogenicity*

No information was available.

2.2.4 *Genotoxicity*

In vitro studies evaluating the potential genotoxicity of cyclotetraglucose are summarized in Table 2. Cyclotetraglucose (pentahydrate) was not mutagenic in several different strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and in *Escherichia coli* WP2uvrA when tested at concentrations of up to 5000 µg/plate with and without metabolic activation (Sokolowski, 2005). Cyclotetraglucose (pentahydrate) was also shown to be non-clastogenic in Chinese hamster V79 cells at concentrations of up to 5000 µg/plate in both the absence and presence of metabolic activation (Schulz, 2005).

2.2.5 *Reproductive toxicity*

No information was available.

2.2.6 *Special studies*

(a) *Ocular irritation and dermal toxicity, irritation and sensitization*

In a study conducted to assess potential dermal toxicity, cyclotetraglucose was dissolved in purified water (0.5 g/ml) and applied topically onto the shaved skin of five male and five female HanBrl:WIST (SPF) rats at a dose level of 4 ml/kg bw (2000 mg cyclotetraglucose/kg bw). A control group was not included. Animals were initially examined for clinical signs of toxicity and mortality at approximately 1, 2, 3

Table 2. Results of in vitro assays for mutagenicity/genotoxicity with cyclotetraglucose

Type of assay	Test system	Concentration	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	0 and 3–5000 µg/plate	Negative[a]	Sokolowski (2005)
Reverse mutation	*Escherichia coli* WP2uvrA	0 and 3–5000 µg/plate	Negative[a]	Sokolowski (2005)
Chromosomal aberration	Chinese hamster V79 cells	0 and 1250–5000 µg/plate	Negative[a]	Schulz (2005)

[a] With and without metabolic activation (rat S9 mix).

and 5 h post-treatment and twice daily thereafter for the duration of the 15-day observation period. Twenty-four hours post-treatment, the semi-occlusive dressing was removed and the treated area was flushed with water, dried and examined macroscopically. Body weights were recorded prior to treatment and on days 8 and 15. At the end of the 15-day observation period, all animals were killed and subjected to gross necropsy. None of the animals died, and no local or systemic signs of toxicity were observed for the duration of the study. Body weights were within the range commonly recorded for this strain and age of rats. Macroscopic findings revealed no treatment-related alterations (Arcelin, 2005).

In additional studies performed to examine the irritation and sensitization potential of cyclotetraglucose, 0.1 g of powdered cyclotetraglucose was shown to be neither irritating nor corrosive after being instilled undiluted into the conjunctival sac of the left eye of New Zealand White rabbits (one male and two females) (Ott, 2003a). Apart from slight reddening, no other abnormal changes of the eye, including ocular discharge or swelling, were observed. In an in vitro assay, cyclotetraglucose demonstrated a protective effect against development of lens opacity of porcine eye lenses incubated in saline solutions for a period of 40 days (Matsuo, 2005). Topical application of 0.5 g cyclotetraglucose moistened with water to the shaved left flank of New Zealand White rabbits (one male and two females) did not cause irritation or other skin reactions as assessed for a period of up to 72 h after treatment (Ott, 2003b). Cyclotetraglucose was not associated with the induction of delayed dermal hypersensitivity following topical rechallenge of a group of 10 male albino guinea-pigs with a 75% aqueous suspension of cyclotetraglucose 3 weeks after initial sensitization consisting of an intradermal injection of 20% cyclotetraglucose aqueous solution followed 1 week later by topical application of a 75% aqueous suspension (Ott, 2004).

(b) Interaction with mineral absorption

Four groups of 10 5-week-old male Wistar rats received a starch-based diet with 0%, 1%, 2% or 5% cyclotetraglucose (anhydrous), which was added to the diets at the expense of starch, for a period of 8 weeks. Based on reported food intake data and body weights, the authors calculated that rats were exposed to daily dose levels of 0, 490, 1010 and 2580 mg cyclotetraglucose/kg bw at the respective dietary concentrations. The diets were also supplemented with 3.5% of a mineral mix containing calcium, magnesium, phosphorus, iron, sodium and potassium. During the last week of the study, 0.5% chromium oxide was added to the diet as a marker of absorption. During the last 2 days of the experiment, the animals were housed in metabolism cages for collection of faeces. At the end of the study period, the animals were killed, and the contents from different segments of the intestine were collected. The intestinal contents and faecal samples were assayed for cyclotetraglucose. Calcium, magnesium, iron, phosphorus and chromium levels were measured in the food, as well as in the intestinal contents and faeces collected from animals. The weight and mineral content (calcium, magnesium and phosphorus) of the femur were also determined. Dose-related increases in the absorption rates of calcium, magnesium, phosphorus and iron were observed along different segments of the gastrointestinal tract compared with the control group. The analysis of the

gastrointestinal contents of all test groups demonstrated that cyclotetraglucose was present in the contents of the ileum; however, no cyclotetraglucose was identified in the appendix and excreted faeces, suggesting that in the more distal segments of the gut, cyclotetraglucose was fermented completely. The weight and calcium, magnesium and phosphorus contents of the femur at all dose levels also increased significantly in response to treatment with cyclotetraglucose (Oku et al., 2006a).

(c) Caecal enlargement

Mice

In 6-week-old BALB/c mice fed diets supplemented with 0%, 1%, 2% or 5% cyclotetraglucose for a period of 4 weeks, the weight of the caecal contents was significantly increased in the high-dose group compared with controls. Based on the reported mean daily food intake and mean initial body weights of the animals, the corresponding daily doses were calculated to be approximately 0, 1860, 3175 and 7480 mg/kg bw per day, respectively. In comparison with the control group, the pH of the caecal contents was decreased in all treated groups; however, only the change in the high-dose group was statistically significant. Although a dose-dependent increase in butyrate levels in the caecal contents was reported relative to the control group, statistical significance was reached only in the high-dose group. Lactate levels in the caecal contents were also increased in all groups of mice administered cyclotetraglucose in their diets compared with controls; however, only the difference in the mid-dose group was statistically significant (Hino et al., 2006).

Rats

In a 28-day feeding study, groups of male Wistar rats (10 per group) were administered 5% cellulose or 1%, 2% or 5% cyclotetraglucose in the diet (approximately 1000, 2000 and 5000 mg/kg bw per day, respectively). The control group was provided a corn starch–based diet. A dose-related increase in the weight of the caecal contents was noted; however, only changes in the 2% and 5% groups were statistically significant in comparison with the corn starch and cellulose groups. Relative to the cellulose group, the pH of the caecal contents was decreased significantly only in the high-dose group. The total amount of organic acids in the caecal contents increased significantly in the high-dose group. The increase in the level of total organic acids was primarily mediated by elevated concentrations of malate, lactate and isovalerate. In comparison with both the corn starch control and cellulose group, the caecal total bile acids content also increased significantly at all dose levels in a dose-dependent manner. A dose-dependent increase was also observed in the total lipids level of the caecal contents in cyclotetraglucose-treated rats, reaching statistical significance compared with both the corn starch control and cellulose groups (Hashimoto et al., 2006).

The weight of the caecal contents and levels of bile acid in the caecum were determined in 5-week-old male Wistar rats administered diets containing 0%, 1%, 2% or 5% spray-dried cyclotetraglucose syrup for a period of 4 weeks. The respective average intake of cyclotetraglucose was reported to be 0, 0.38, 0.81 and 2.05 g/kg bw per day. In comparison with the control group, weights of the caecal

contents were significantly increased in the mid- and high-dose groups, whereas levels of bile acid in the caecal contents were increased at all dose levels (Oku et al., 2006b).

(d) Reduced body fat deposition

Rats

In a 28-day feeding study, groups of 10 5-week-old male Wistar rats were provided a non-fibre corn starch control diet or test diets with 5% cellulose or 1%, 2% or 5% cyclotetraglucose (approximately 0, 1000, 2000 and 5000 mg/kg bw per day, respectively) in place of an equivalent amount of corn starch. Body weights, as well as serum and liver lipids, liver enzymes and the weights of intestinal, perirenal and epididymal fat pads, were measured. Two rats from the high-dose group were reported to have soft stools for the first 3 days of the study period; however, laxation was not observed in any of the groups for the duration of the study. Final body weight, body weight gain and food utilization efficiency of the animals in the high-dose group and food utilization efficiency of low-dose animals were significantly lower in comparison with the cellulose group. Significant reductions in body weight gain and food utilization efficiency were also observed in the high-dose rats relative to the corn starch control group. In comparison with both the corn starch control and cellulose groups, weights of the intestinal, perirenal and epididymal fat pads were decreased significantly in animals at all dose levels, with the exception of the intestinal fat weight in the low-dose group. Serum triglyceride levels also decreased significantly in the mid- and high-dose groups compared with both the corn starch control and cellulose groups, whereas levels of liver triglycerides were significantly reduced only in the low-dose group compared with both the corn starch control and cellulose groups and in the mid-dose group compared with the corn starch control. Serum total cholesterol and phospholipid levels in high-dose animals were also significantly reduced in comparison with the corn starch control group. Apart from an incidental decrease in the absolute liver weight of rats in the low-dose group compared with the corn starch controls, no treatment-related effects were observed in liver weight and levels of liver enzymes, glucose-6-phosphate dehydrogenase and acyl-coenzyme A oxidase (Hashimoto et al., 2006).

A rat feeding study that was performed using spray-dried cyclotetraglucose syrup produced similar results. Five-week-old male Wistar rats were administered diets containing 0%, 1%, 2% or 5% spray-dried cyclotetraglucose syrup for a period of 4 weeks. The respective average intake of cyclotetraglucose was reported to be 0, 0.38, 0.81 and 2.05 g/kg bw per day. Relative to the control group, serum total and low-density lipoprotein cholesterol levels in all groups of test animals, high-density lipoprotein cholesterol and phospholipid levels in the low- and high-dose animals, and levels of triglycerides and glutamate–oxaloacetate transaminase in the high-dose animals were significantly decreased. In comparison with the control group, liver triglyceride levels were reduced significantly in the mid- and high-dose groups, whereas liver weights and total liver lipids and cholesterol were unaffected at all dose levels. The renal, intestinal and testicular fat deposits were reduced significantly in the mid- and high-dose groups (Oku et al., 2006b).

Additional feeding studies in which rats received a diet high in fat and sucrose, which was unsupplemented (control 1), supplemented with cellulose (control 2) or supplemented with up to 5% cyclotetraglucose or spray-dried cyclotetraglucose syrup, produced similar results (Kurose, 2004a, 2004b). In a 12-day preliminary study, 8-week-old male Wistar rats were fed a non-fibre diet (AIN-93G diet with cellulose removed) or a high-fat/high-sucrose diet (non-fibre diet with soybean oil exchanged with lard and increased sucrose content) supplemented with 0% or 5% cyclotetraglucose (approximately 0 and 5000 mg/kg bw per day, respectively). Diets were labelled as non-fibre control, high-fat/high-sucrose control and treatment, respectively. Relative to the high-fat/high-sucrose control group, the renal, intestinal and testicular fat deposits were significantly reduced in the treatment group (fat weights of the treatment group were comparable with those of the non-fibre control group). Serum analyses revealed significantly lower triglyceride levels in the treated group in comparison with the high-fat/high-sucrose control animals (treatment triglyceride levels were comparable with those of the non-fibre control groups), whereas fat analyses of the liver revealed no significant variations among any of the study groups. In comparison with the non-fibre control, body weight gains were significantly greater in the high-fat/high-sucrose control and treatment groups, although no difference in food intake was reported among any of the groups (Kurose, 2004a).

In a subsequent 4-week study, male Wistar rats were fed a non-fibre diet (AIN-93G diet with cellulose removed) or a high-fat/high-sucrose diet (non-fibre diet with soybean oil exchanged with lard and increased sucrose content) supplemented with 0%, 1%, 2% or 5% cyclotetraglucose (approximately 0, 1000, 2000 and 5000 mg/kg bw per day, respectively) (labelled as non-fibre control, high-fat/high-sucrose control, low-, mid- or high-dose treated groups, respectively). A high-fat/high-sucrose diet supplemented with cellulose was also included. Fat weight values for all cyclotetraglucose-treated animals were comparable with or slightly lower than those for the non-fibre control group. Following 2 weeks of treatment, triglyceride levels of the mid- and high-dose treatment groups were comparable with those of the non-fibre control group. After 4 weeks, triglyceride levels in the high-dose group were significantly lower than those of the non-fibre control group. Fat analyses of the liver revealed lower phospholipid levels (statistical significance not reported) in all cyclotetraglucose-treated groups in comparison with the non-fibre control group. Some of the animals of the high-dose group (number not specified) exhibited loose stools, but were reported to recover “one week after”, presumably, treatment cessation (Kurose, 2004b).

Hamsters

The potential effect of cyclotetraglucose or cyclotetraglucose syrup on fat metabolism was also evaluated in 36 male Syrian hamsters (six per group). The hamsters were provided a non-high-fat diet (control) or high-fat diets that were unsupplemented (high-fat diet control) or supplemented with 2% or 5% cyclotetraglucose or 4% or 11% cyclotetraglucose syrup (diets 1, 2, 3, 4, 5 and 6, respectively) (the exact duration of the study period was not specified in the English-language abstract, but appeared to be longer than 6 weeks, based on figures and

tables). Body weight gains were comparable across all high-fat diet groups (i.e. groups 2–6). Food intakes in groups 3–5 were increased (statistical significance not reported) in comparison with the control group (diet 1), but were comparable to the food intake of group 2. No differences in the fat around the liver, kidney and testis were observed. No differences among all study groups were reported with respect to plasma total cholesterol and triglyceride levels measured at 2, 4 and 6 weeks of the study. None of the animals were observed to experience loose stools (Inoue, 2005).

2.2.7 Data pertaining to the safety of the enzymes used in the production of cyclotetraglucose and other potential impurities

CGTase (EC 2.4.1.19), which is used in the production of cyclotetraglucose and cyclotetraglucose syrup, is obtained from a strain of *Bacillus stearothermophilus*. Data on the toxicity of CGTase from other sources were considered previously by the Committee in the context of the assessments of the safety of α-cyclodextrin, β-cyclodextrin and γ-cyclodextrin, and no concern was raised (Annex 1, references *107*, *116*, *138* and *155*). The safety of *B. stearothermophilus* as a source organism was considered previously in the context of the evaluation of the safety of α-amylase from this organism (Annex 1, reference *94*), in which it was concluded that *B. stearothermophilus* is non-pathogenic to humans and animals. Based on the previous evaluations of the safety of *B. stearothermophilus*, the Committee also expressed no concern over the use of CGTase from *B. stearothermophilus* in the production of trehalose (Annex 1, reference *150*).

The mixture of enzymes 6-GT and IMT is derived from *Bacillus globisporus* N75 without any genetic modification. The first strains of *B. globisporus* that were shown to be capable of converting starch to cyclotetraglucose, strains C9 and C11, were isolated from soil (Aga et al., 2002a, 2002b; Nishimoto et al., 2002). Aga et al. (2003, 2004) reported that further screening of soil bacteria revealed a separate isolate, *B. globisporus* N75, to be a producer of 6-GT and IMT, but with a higher thermal stability. Both enzymes are secreted by *B. globisporus* into the extracellular environment (Nishimoto et al., 2002; Aga et al., 2003). Thus, for the production of the enzyme preparation, *B. globisporus* N75 is grown in a culture medium containing food-grade dextrin, yeast extract, potassium and sodium phosphate, magnesium sulfate and calcium carbonate at temperatures of 27–30 °C (optimal growth temperature) (Aga et al., 2003). When the desired enzyme activity has been attained in the culture broth, the cells are removed by filtration through a microfilter membrane. The filtrate is subsequently purified and concentrated by ultrafiltration. Approximately 50% of the dry substance represents protein that can be precipitated with saturated ammonium sulfate. The ultrafiltered culture supernatant possesses specific 6-GT and IMT activities of 100 and 320 U/g (on dry basis), respectively (Aga et al., 2003).

Bacillus globisporus was first described as a *Bacillus* species by Larkin & Stokes (1967) during an investigation of psychrophylic strains of *Bacillus*. Since then, different strains of *B. globisporus* have been isolated from soil and river water (Larkin & Stokes, 1967; Rüger, 1983; Bandyopadhyay et al., 1993). On the basis of

a recent taxonomic re-evaluation, it has been proposed to transfer *B. globisporus* to the genus *Sporosarcina* (*Sporosarcina globispora*) (Yoon et al., 2001). Accordingly, the strains deposited originally as *B. globisporus* in the American Type Culture Collection, Deutsche Sammlung von Mikroorganismen und Zelkulturen GmbH and other culture type collections are now listed under *Sporosarcina globispora* (Euzéby & Tindall, 2004). The cells of *B. globisporus* have a rod-like shape and peritrichous flagella. They grow well at temperatures of 25 ºC, with spore formation occurring at 0 ºC. *Bacillus globisporus* is listed in risk group 1 of the German Committee for Biological Agents (Ausschuss für Biologische Arbeitsstoffe, 2006). The potential virulence of different bacteria, including one strain of *B. globisporus*, isolated from tap water was examined by Payment et al. (1994). In line with earlier observations (Rüger, 1983), *B. globisporus* did not grow at temperatures of 35 ºC or greater and did not exhibit any virulence factors (e.g. haemolysis, cell adherence, cell invasion), thus largely limiting its potential for pathogenicity in humans.

2.3 Observations in humans

2.3.1 Cyclotetraglucose

In a crossover trial, aqueous solutions of cyclotetraglucose (pentahydrate) were provided to a group of 28 healthy, predominantly male human volunteers (20 males and 8 females) at dose levels of 5, 10, 20 or 30 g for oral consumption not less than 2 h after lunch or dinner. The lowest dose was administered first, and subsequent doses were administered in a blinded fashion. Tetraitol syrup was administered as a control. A washout period of over 3 days between each testing interval was reported. Two subjects (one male and one female) dropped out during the study period: one of the subjects was reported to develop a cold, whereas the other reported constipation as the reason for early withdrawal. From questionnaires that subjects completed within 24 h of each treatment, dose-dependent increases were observed in abdominal complaints (i.e. abdominal distension and sounds and flatulence); however, none of the subjects developed a fever or experienced any adverse effects. Five of the 26 subjects (3 males and 2 females) reported laxative effects after the consumption of 20 g cyclotetraglucose. At the next and highest dose level tested (30 g), 12 of the 26 subjects (10 males and 2 females) experienced laxation. The author indicated that the reported symptoms were comparable with symptoms experienced following administration of other saccharides of limited digestibility (Miwa, 2002).

2.3.2 Cyclotetraglucose syrup

The intestinal tolerance of cyclotetraglucose syrup was examined in a group of 40 healthy, predominantly male human volunteers (28 males and 12 females) in a double-blind crossover study. Subjects were provided the test product at dose levels of 25, 40, 55 or 70 g (dry matter basis) (the time between each treatment was not reported). A readily digestible, maltotetraose syrup was administered as a negative control. One female subject dropped out prior to completing the

study because of a cold. Laxation was reported to occur in 1, 5 and 25 of the 39 subjects at the 40, 55 and 70 g cyclotetraglucose dose levels, respectively (Miwa et al., 2004).

3. DIETARY EXPOSURE

Cyclotetraglucose and cyclotetraglucose syrup are intended to be used as carriers for flavours and certain nutritive substances, such as polyunsaturated fatty acids and certain vitamins, and as a food ingredient (dietary fibre). The proposed maximum use levels for cyclotetraglucose and cyclotetraglucose syrup in a variety of different foods are presented in Table 3 and are representative of their use as food ingredients (dietary fibre). Use levels as carriers are expected to be significantly lower and so are incorporated in the proposed levels for use as dietary fibre, as they are likely to be used in similar food groups (Bär, 2006).

Table 3. Proposed maximum use of cyclotetraglucose/cyclotetraglucose syrup

Food category	Food use	Maximum use level[a] (%)
Bakery products	Breads and rolls, cakes (light weight), quick breads, dough (refrigerated), baking mixes	5
	Brownies, bars (grain based)	7
	Crackers (sweet and non-sweet)	10
Beverages	Beverage mixes, diet soft drinks	1 (prepared)
	Fruit juices	1
	Vegetable juices	2
	Instant coffee/tea, coffee whitener	1 (dry)
	Formula diets	1 (prepared)
	Soy and non-soy (imitation) milk	2 (prepared)
Cereals and other grain products	Ready-to-eat breakfast cereals	2–9
	Instant rice, pasta and noodles	2
Condiments	Condiments	3
Dairy products	Yoghurt, frozen dairy products	2.5
	Pudding mixes	1 (dry)
	Milk beverage mixes	2.5 (prepared)
Fats and oils	Reduced fat spreads	20
	Dressing and mayonnaise	5
Snacks	Salty snacks	1
Soups	Canned soups, dry soups	2 (prepared)
Sugars and sweets	Hard candy	15

[a] Percentage refers to cyclotetraglucose or cyclotetraglucose syrup (solids).

Naturally occurring levels of cyclotetraglucose in foods, such as sake, sake lees and yeast, were not taken into consideration in any of the intake estimates considered by the Committee. Concentrations in sake and sake lees have been reported at 0.2–7 µg/g and 60–380 µg/g, respectively (Watanabe et al., 2004), well below the proposed levels of additions to other foods (1% or more). However, sake is not commonly consumed in the countries assessed and was therefore considered unlikely to make a significant contribution to total intakes of cyclotetraglucose. This is also expected to be the case in relation to yeast. Whereas no data were available on naturally occurring concentrations in yeast, the amount of yeast consumed would not be expected to result in this food ingredient being a significant contributor to total cyclotetraglucose intakes.

3.1 Single-day intake estimates

An intake assessment for the United States of America (USA) formed part of the submission from the sponsor (Bär, 2006). Food consumption data for the national population aged 2 years and over were based on individual dietary records from continuing surveys of food consumption in 1994–1996 and 1998, averaged over 2 days of data (United States Department of Agriculture, 1994–1996, 1998). These data were combined with the predicted use of cyclotetraglucose and cyclotetraglucose syrup in a wide range of foods at the maximum use levels (Table 3) to predict intakes of cyclotetraglucose and cyclotetraglucose syrup for the population of the USA (Table 4).

The mean daily intake of cyclotetraglucose or cyclotetraglucose syrup for the total population of the USA (2 years and above) was estimated to be 11.8 g/person or 210 mg/kg bw (Table 4). The 90th percentile intake was estimated to be 19.8 g/person per day or 430 mg/kg bw per day. For population subgroups, the highest mean and 90th percentile intakes for all foods combined were estimated for the 13- to 19-year age group (12.4 and 21.4 g/person per day, respectively). However, on a per kilogram of body weight basis, the highest mean and 90th percentile intake estimates were reported for the 2- to 5-year age group (610 and 980 mg/kg bw per day, respectively).

Intakes of cyclotetraglucose and cyclotetraglucose syrup for Australia and New Zealand were estimated during the Committee's assessment, based on 24-h recall food consumption data for a single day from the Australian 1995 (McLennan & Podger, 1998) and New Zealand 1997 (Russell et al., 1999) National Nutrition Surveys and predicted use of cyclotetraglucose and cyclotetraglucose syrup in a wide range of foods at the maximum use levels (Tables 3 and 4). For the Australian population aged 2 years and over, mean daily intakes were predicted to be 14.3 g/person (260 mg/kg bw), and 90th percentile intakes, 25.5 g/person per day (525 mg/kg bw per day). For the New Zealand population aged 15 years and over, mean daily intakes were predicted to be 14.8 g/person (205 mg/kg bw), and 90th percentile intakes, 26.6 g/person per day (375 mg/kg bw per day).

Predicted intakes of cyclotetraglucose or cyclotetraglucose syrup for Australia and New Zealand were in the same range as those predicted for the population in the USA, but slightly higher at the mean and 90th percentile, as might

Table 4. Estimated daily intakes for consumers of foods proposed to contain cyclotetraglucose/cyclotetraglucose syrup (dry basis)

Country	Survey (year)	Assumptions	Model	Intake in g/day (mg/kg bw per day)
USA	2 × 24-h recall, 2-day average food consumption data USDA CSFII 1994–1996, all ages excluding records for pregnant and lactating women Supplementary children's survey, USDA CSFII 1998 Sample size 20 600, 84% consumers (17 260) Sample size for age groups: 5435 (2–5 years), 2089 (6–12 years), 1211 (13–19 years), 9189 (20+ years)	Proposed levels (Table 3) Present in all foods in nominated food groups	Mean all 90th percentile Mean 2–5 years 90th percentile Mean 6–12 years 90th percentile Mean 13–19 years 90th percentile Mean 20+ years 90th percentile	11.8 (210) 19.8 (430) 10.2 (610) 16.2 (980) 11.8 (380) 18.7 (650) 12.4 (200) 21.4 (360) 11.3 (160) 20.2 (280)
Australia	1995 NNS, 2 years and over Sample size 13 858, 99.8% consumers (13 829) Single-day food consumption, 24-h recall	Proposed levels (Table 3) Present in all foods in nominated food groups	Mean all 90th percentile	14.3 (260) 25.5 (525)
New Zealand	1997 NNS, 15 years and over Sample size 4636, 99.7% consumers (4622) Single-day food consumption, 24-h recall	Proposed levels (Table 3) Present in all foods in nominated food groups	Mean all 90th percentile	14.8 (205) 26.6 (375)

CSFII, Continuing Survey of Food Intakes by Individuals; NNS, National Nutrition Survey; USDA, United States Department of Agriculture.

be expected, as a single day's food consumption data were used in these calculations compared with the 2-day average of foods consumed that was used for the United States estimates. Minor differences in the food classification systems used may also have contributed to differences in results, as well as different food consumption patterns in each country.

3.2 Single food or meal intake estimates

Estimated intakes of cyclotetraglucose or cyclotetraglucose syrup were also calculated per eating occasion (e.g. breakfast, lunch, dinner, supper, snack) and by food group per eating occasion for the population in the USA to determine whether a single meal or a single food at a single meal, particularly eaten in a large amount, could result in adverse side-effects attributed to a bolus dose. The highest predicted intake of cyclotetraglucose or cyclotetraglucose syrup from all foods at any single

eating occasion was 10.4 g/person at dinner for 13- to 19-year-olds. The highest intake (90th percentile) on a single eating occasion predicted for any single food for all age groups assessed was 14 g of cyclotetraglucose or cyclotetraglucose syrup from soups for 13- to 19-year-olds.

Although not directly comparable, intakes of cyclotetraglucose or cyclotetraglucose syrup per food group per day rather than per eating occasion were predicted for Australia and New Zealand populations using both mean and 90th percentile consumption data for consumers only of each food group. For Australia, all single-food daily intakes were 16 g/day or below; for New Zealand, all single-food daily intakes were 15 g/day or below. The highest intakes were predicted to be from soy beverages.

4. COMMENTS

4.1 Toxicological data

Studies in animals and humans consistently indicate that cyclotetraglucose largely escapes hydrolysis and absorption in the upper gastrointestinal tract and is only slowly degraded by the intestinal microflora. In vitro, cyclotetraglucose was not hydrolysed by human salivary or porcine pancreatic α-amylase or by artificial gastric juice preparations, and less than 1% incurred ring opening in the presence of rat intestinal mucosa to form a linear saccharide.

The small fraction that may be subject to enzymatic hydrolysis is expected to be absorbed as glucose. However, 12 h following administration of cyclotetraglucose (100 mg/kg bw) to rats via oral gavage, 94% of the administered dose was collected in the faeces, 6% was identified in the gastrointestinal tract and none was detected in blood, indicating that cyclotetraglucose was not metabolized or absorbed in rats. Although no studies on the metabolic fate of cyclotetraglucose in humans were available, the absence of an increase in plasma glucose and insulin levels following consumption of cyclotetraglucose provides indirect evidence that cyclotetraglucose was not hydrolysed to glucose. Furthermore, cyclotetraglucose was not detected in blood. Conversely, glycaemic and insulinaemic responses were observed in humans ingesting cyclotetraglucose syrup, likely due to the presence of digestible linear carbohydrate components in the syrup.

Following incubation with human stool samples for 24 h, fermentation of cyclotetraglucose was demonstrated to be highly variable, ranging from <5% to 25% within the first 6 h and from <5% to 100% by the end of the 24-h incubation period. A similarly variable fermentation profile was reported for cyclotetraglucose syrup.

A number of acute and short-term toxicity studies were reviewed, which indicated low toxicity by the oral route. No adverse effects were reported in groups of male and female rats given single doses of cyclotetraglucose or cyclotetraglucose syrup at up to 5000 mg/kg bw. Likewise, cyclotetraglucose was not associated with any toxicity in rats following single-dose (2000 mg/kg bw) topical application. The results of 90-day rat toxicity studies with cyclotetraglucose as well as cyclotetraglucose syrup indicated that dietary concentrations of up to 10%

(approximately 7000 mg/kg bw) were not associated with any toxicologically significant adverse effects. The results of in vitro genotoxicity assays were negative. No long-term studies of toxicity, reproductive/developmental toxicity or carcinogenicity have been conducted with cyclotetraglucose; however, the Committee concluded that given the known fate of the compound in the gastrointestinal tract, such studies were not required for an evaluation.

Cyclotetraglucose was shown not to possess ocular or dermal irritating or sensitization properties when applied undiluted or moistened with water. Rats given oral cyclotetraglucose doses of up to 2580 mg/kg bw showed increased absorption of minerals (calcium, magnesium, iron and phosphorus). In studies conducted specifically to assess the potential for caecal enlargement as a result of cyclotetraglucose consumption, an effect commonly observed with non-digestible materials, increases in the weight of the caecal contents were observed in both mice and rats following dietary administration of cyclotetraglucose or spray-dried cyclotetraglucose syrup at doses ranging from 5000 to 7500 mg/kg bw. Increases in caecal content weights were accompanied by decreased caecal pH levels and increases in short-chain fatty acid and bile acid levels, effects that are typically encountered with materials that traverse the upper segments of the gastrointestinal tract without digestion, but are fermented in the colon. Additionally, rats maintained on cyclotetraglucose-supplemented diets generally exhibited reduced organ fat content, together with reductions in serum cholesterol and triglyceride levels. These effects were not observed in hamsters.

In a multiple dose level, single-administration human tolerance trial involving 28 adults, a dose-dependent increase was observed in the incidence of gastrointestinal complaints following consumption of single doses of cyclotetraglucose solutions. None of the study subjects experienced laxation after ingestion of 5 or 10 g of the cyclotetraglucose solution; however, at the two higher dose levels evaluated (20 and 30 g), dose-related increases were noted in the occurrence of laxation. In a clinical study involving 40 adults, laxation was reported at dose levels of 40 g of cyclotetraglucose syrup and greater (55 and 70 g), with incidences increasing in proportion to the dose level of the syrup. No laxative effects were reported following consumption of 25 g of cyclotetraglucose syrup. The difference in laxative properties of cyclotetraglucose and cyclotetraglucose syrup is due to the fact that the syrup contains only about 30–40% cyclotetraglucose. No studies involving repeat administration of cyclotetraglucose were provided to the Committee for review.

The 6-GT/IMT enzyme preparation, which is used in the production of cyclotetraglucose and cyclotetraglucose syrup, was reported to be derived from a strain N75 isolated from a soil sample and identified as belonging to *Bacillus globisporus* (currently *Sporosarcina globispora*). No information was provided to support this classification, nor were data provided on the pathogenicity or toxigenicity of the source organism.

4.2 Assessment of dietary exposure

The predicted daily dietary exposures to cyclotetraglucose or cyclotetraglucose syrup for consumers only were based on food consumption data derived from individual dietary records reported in the 1994–1998 surveys for the USA and proposed maximum levels of use in a variety of foods as food ingredients, assuming that these levels encompassed the much lower levels of use as a carrier and stabilizer. Dietary exposure estimates from naturally occurring sources, such as sake, were not included in this assessment, as these sources are not commonly consumed by the population in the USA. The contribution from yeast was not considered to be of significance. The mean daily dietary exposure estimate for consumers only in the United States population aged 2 years and above was 12 g/day, and the 90th percentile dietary exposure was 20 g/day (210 and 430 mg/kg bw per day, respectively). The highest dietary exposures were predicted for 13- to 19-year-olds, whose mean and 90th percentile dietary exposures were 12 and 21 g/day, respectively. On a body weight basis, children aged 2–5 years had the highest predicted mean and 90th percentile dietary exposures for cyclotetraglucose or cyclotetraglucose syrup (610 and 980 mg/kg bw per day, respectively). Similar levels of dietary exposure were predicted for Australian and New Zealand populations. The highest predicted dietary exposure to cyclotetraglucose or cyclotetraglucose syrup at a single eating occasion was 10 g/person and from a single food 14 g/person, both figures for 13- to 19-year-olds in the population in the USA.

5. EVALUATION

Cyclotetraglucose was listed on the agenda for evaluation as a stabilizer and carrier; however, it was brought to the attention of the Committee that cyclotetraglucose may have additional uses as a food ingredient. The Committee concluded that the existing data are adequate to support the safety of cyclotetraglucose and cyclotetraglucose syrup, provided that data are submitted to the Committee regarding the identity of the bacterial strain used to produce the 6-GT/IMT enzyme preparation and evidence of its lack of pathogenicity and toxigenicity. A temporary acceptable daily intake (ADI) “not specified” was allocated for cyclotetraglucose and cyclotetraglucose syrup pending submission of these additional data.

Although the highest predicted dietary exposure from a single eating occasion is lower than the laxative dose of approximately 20 g/day, the Committee noted that laxative effects should be taken into account when considering appropriate levels of use of cyclotetraglucose or cyclotetraglucose syrup as food ingredients.

The specifications for the syrup were made tentative pending submission of further information on the total saccharide content and test methods and the unidentified saccharide fraction. The temporary ADI for cyclotetraglucose and cyclotetraglucose syrup and the tentative specifications for cyclotetraglucose syrup will be withdrawn if the requested data are not received by the end of 2008.

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ISOAMYLASE FROM PSEUDOMONAS AMYLODERAMOSA

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1. EXPLANATION

At the request of the Codex Committee on Food Additives and Contaminants at its thirty-eighth session (Codex Alimentarius Commission, 2006), the Committee evaluated the enzyme isoamylase (glycogen α-1,6-glucanohydrolase; EC 3.2.1.68). Isoamylase catalyses the hydrolysis of 1,6-α-D-glucosidic branch linkages in glycogen, amylopectin and their beta-limit dextrins. It has no or only limited activity on linear polysaccharides linked by α-1,6-glycosidic bonds (e.g. pullulan) and on alpha-limit dextrins.

The isoamylase enzyme preparation that was evaluated is obtained by pure culture fermentation of *Pseudomonas amyloderamosa*. The Committee reviewed toxicological data on this enzyme at its fifty-fifth meeting (Annex 1, reference *149*) as part of the safety assessment of trehalose. The Committee at that meeting concluded that the available data on isoamylase (i.e. a study of acute toxicity, a 13-week study of toxicity and a bacterial mutagenicity assay) did not raise any safety concern.

The enzymatic activity of isoamylase is determined by measuring the rate of hydrolysis of high-amylopectin starch and is expressed in isoamylase units (IAU). The isoamylase enzyme preparations contain at least 1.25×10^6 IAU/g and have the following composition: total organic solids (TOS), approximately 4%; water, approximately 58%; maltose and glucose, approximately 37%; and glycerol fatty acids, approximately 0.1%.

Isoamylase is used primarily in the production of food ingredients from starch (e.g. glucose syrup, maltose and maltitol, trehalose, cyclodextrins and resistant starch). It is typically used in combination with other amylolytic enzymes, such as α-amylase, β-amylase and glucoamylase, which further degrade the linear dextrins that arise from the debranching activity of isoamylase. The recommended use levels range from 50 to 5000 IAU/g starch.

1.1 Production strain

The production strain is *P. amyloderamosa* MU 1174. This strain was derived from the isoamylase-hyperproducing strain MI 414, which was obtained from strain SB-15 (ATCC 21262), a wild-type strain of *P. amyloderamosa* isolated from soil, by means of chemical mutagenesis (using *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine) and selection procedures.

1.2 Product characterization

Isoamylase is produced by pure culture fermentation of the production strain *P. amyloderamosa* MU 1174. It is secreted into the fermentation medium, from which it is recovered and concentrated. It is subsequently stabilized, formulated and standardized with maltose, glucose, water and either glycerol fatty acids or sodium benzoate. In the preparation named “Isoamylase M”, glycerol *n*-caproate and glycerol octylate are used; in “Isoamylase S”, sodium benzoate is used.

Compared with the isoamylase activity, the activity of other hydrolytic enzymes (cellulase, lipase and protease) is very low (<0.06%) in the isoamylase enzyme preparation. When three batches of the isoamylase enzyme preparation were analysed for aflatoxins (B_1, B_2, G_1 and G_2), sterigmatocystin, zearalenone and ochratoxin, none of these mycotoxins was detected.

The isoamylase enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing, prepared by the Committee at its sixty-seventh meeting (Annex 1, reference *184*).

The isoamylase enzyme preparation from *P. amyloderamosa* has been used in the food industry in countries such as Japan for more than 20 years. When used

in the production of starch-derived food ingredients, isoamylase is usually inactivated (typically by heat treatment) or removed at the end of the production processes. Therefore, the final products no longer contain isoamylase activity.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

No information was available.

2.2 Toxicological studies

The source organism *P. amyloderamosa* is non-pathogenic and non-toxigenic (see also section 2.2.6) and has a listing in the American Type Culture Collection as having no known potential to cause disease in humans or animals (BioSafety Level 1).

2.2.1 Acute toxicity

The acute oral toxicity of isoamylase from *P. amyloderamosa* (no further specifications given) was examined in one study in ddy-N mice (10 per sex per group). This study was also reviewed by the Committee at its fifty-fifth meeting (Annex 1, reference *149*). The LD_{50} was >17 g/kg body weight (bw) (Morimoto et al., 1979a).

2.2.2 Short-term studies of toxicity

In a 13-week study of toxicity, which was also reviewed by the Committee at its fifty-fifth meeting (Annex 1, reference *149*), groups of 20 male and 20 female Wistar rats (aged 4–5 weeks) were given isoamylase from *P. amyloderamosa* (batch 80519; stabilized solution containing 19 mg protein/g, equivalent to 1 362 500 IAU/g; TOS content, 3.1% weight per weight; specific gravity, 1.2 g/ml) by gavage at a dose equivalent to 0, 90, 190 or 370 mg TOS/kg bw per day. The study was performed according to international guidelines and was certified for compliance with Good Laboratory Practice (GLP) and quality assurance (QA). The animals were examined throughout the study for clinical signs, and body weights were measured weekly. Blood was collected at the end of the study for haematology and blood chemistry, and urinary analysis was conducted. Ophthalmoscopic examinations were performed on control and high-dose animals at the beginning and end of the study. All animals were necropsied at 3 months, and selected tissues were taken for microscopic examination.

Six animals died during the study, their deaths related to the dosing procedure rather than to the test substance. The results of the ophthalmoscopic examinations were normal. There were no treatment-related changes in clinical signs and mean body weight, and food and water consumption were normal in all groups. A statistically significant increase in haemoglobin concentration was found in females at the low and high doses, but the absence of a dose–response relationship and of other haematological changes suggests that these changes

were not treatment related. There were no significant differences in clinical chemical or urinary parameters between the control and treated groups. At necropsy, no treatment-related changes in absolute or relative organ weights were found, and the only gross pathological changes seen were those associated with gavage. Histopathological examination revealed no changes associated with treatment. The no-observed-effect level (NOEL) is 370 mg TOS/kg bw per day, the highest dose tested (Lina, 2000).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.2.4 Genotoxicity

The results of two studies of genotoxicity with isoamylase from *P. amyloderamosa* in vitro are summarized in Table 1. The first study, which was also reviewed by the Committee at its fifty-fifth meeting (Annex 1, reference *149*), was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (1997) and used batch 90308, an unstabilized aqueous solution containing 19 g protein/g (density, 1.01–1.02 g/l). The second study was performed according to OECD Test Guideline 473 (1997) and used batch 00426 (no further details given). Both studies were certified for compliance with GLP and QA.

2.2.5 Reproductive toxicity

No information was available.

2.2.6 Special studies: Pseudomonas amyloderamosa

(a) Toxicity

The acute oral toxicity of *P. amyloderamosa* was examined in two studies in ddy-N mice (10 per sex per group). *Pseudomonas amyloderamosa* was given as wet cells in one study and as culture filtrate in the other (no further specifications given). The LD_{50} values were >66 and >60 g/kg bw, respectively (Morimoto et al., 1979b, 1979c).

(b) Pathogenicity

The pathogenicity of *P. amyloderamosa* was tested in NMRI Han mice (five per sex per group) following a single intravenous injection of 0, 4.35×10^8, 4.35×10^9 or 4.35×10^{10} colony forming units/kg bw. The study was certified for GLP and QA. *Pseudomonas amyloderamosa* was not pathogenic in this study, as demonstrated by the absence of mortality and treatment-related clinical signs and macroscopic abnormalities. Histopathology on liver, kidneys and brain, which on microbiological count did not contain any *P. amyloderamosa*, also did not reveal treatment-related abnormalities (Dufour, 2000).

Table 1. Genotoxicity of isoamylase in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537; *Escherichia coli* WP2uvrA	62–5000 µg protein/plate	Negative[a]	Van Delft (1999)
Chromosomal aberration	Human lymphocytes	1st experiment: 2500, 3750 or 5000 µg/ml, ±S9 2nd experiment: 1250, 2500 or 5000 µg/ml, ±S9	Negative[b]	Haddouk (2001)

S9, 9000 × *g* supernatant from rat liver.

[a] With and without metabolic activation (S9), by the direct plate incorporation method.

[b] With and without metabolic activation (S9). In the first experiment, the cell cultures were treated for 3 h without and with S9 and were harvested 17 h later. The highest tested concentration induced 19% and 10% mitotic inhibition in the absence and presence of S9, respectively. In the second experiment, the cells were exposed continuously without S9 or for 3 h with S9 and were harvested at 20 and, at 5000 µg/ml only, 44 h after start of treatment. The highest tested concentration induced 0% and 15% mitotic inhibition in the absence of S9 at 20 and 44 h, respectively, and 39% and 29% mitotic inhibition in the presence of S9 at 20 and 44 h, respectively.

2.3 Observations in humans

No information was available.

3. DIETARY EXPOSURE

Isoamylase is used for the production of food ingredients from starch. Although at the end of the production processes the enzyme is usually inactivated or removed, the actual levels of the enzyme (active or inactive) in the final food products are not known. A worst-case scenario for human dietary exposure can be estimated on the basis of the recommended use levels and the assumption that all TOS originating from the enzyme preparation are carried over into the final products. To elaborate this scenario, it is assumed that:

- all food ingredients are produced from starch processes using the isoamylase enzyme preparation at the highest recommended use level;
- at maximum, 5000 IAU are needed for treating 1 g starch;
- 1 IAU is contained in approximately 0.03 µg TOS;
- all TOS are carried over into the final products.

The resulting maximum concentration of enzyme in the final products would correspond to 0.15 mg TOS/g of food.

Regarding the consumption of food ingredients from starch, an accurate estimate is problematic, because those ingredients are not usually recorded in food consumption surveys. Based on the highest value for flour consumption from the Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme (GEMS/Food) Consumption Cluster Diets (400 g, Cluster B) and the assumption of a maximum transformation factor of 80% from flour to starch, the resulting daily consumption of starch is 320 g.

In a previous evaluation of the safety of another amylase used in food (Annex 1, reference *167*), the Committee used the budget method (Hansen, 1979) and the worst-case scenario that 20% of calories in the diet are from starch-derived carbohydrates, all of which are consumed. The upper physiological intake of calories according to the budget method is 100 kcal/kg bw per day. The ingestion of starch-derived carbohydrates would therefore be 20 kcal/kg bw per day or, with 4 kcal provided by 1 g carbohydrates, 5 g/kg bw per day, corresponding to 300 g/day for an individual with a body weight of 60 kg. This result is confirmed by an estimate of starch consumption in France, where, based on individual food consumption surveys, the consumption of starch for adult men was estimated to be 161 g/day and 285 g/day for the mean and the 95th percentile, respectively (Volatier, 2000).

The combination of the maximum concentration of 0.15 mg TOS/g food with a daily consumption of 320 g of starch-based food results in a conservative dietary exposure of 48 mg TOS/day, or 0.8 mg TOS/kg bw per day, assuming a body weight of 60 kg.

4. COMMENTS

4.1 Toxicological data

Toxicological studies were performed with an isoamylase liquid enzyme concentrate (LEC). The Committee noted that the materials added to the isoamylase LEC for stabilization, formulation and standardization either have been evaluated previously by the Committee or are common food constituents and do not raise safety concerns.

In a 13-week study of toxicity in rats, no significant treatment-related effects were seen when the stabilized LEC was administered by oral gavage at doses up to and including 370 mg TOS/kg bw per day. Therefore, 370 mg TOS/kg bw per day, the highest dose tested, was taken to be the NOEL. The LEC was not mutagenic in an assay for mutagenicity in bacteria in vitro and was not clastogenic in an assay for chromosomal aberrations in mammalian cells in vitro.

4.2 Assessment of dietary exposure

Based on a maximum mean daily consumption of 320 g of starch-derived carbohydrates (GEMS/Food Consumption Cluster Diet B) by a 60-kg adult and on the assumptions that the enzyme is used at the maximum recommended use level

and that all TOS originating from the enzyme preparation are carried over into the final products, the dietary exposure would be 0.8 mg TOS/kg bw per day.

5. EVALUATION

Comparing the conservative exposure estimate with the NOEL from the 13-week study of oral toxicity, the margin of safety is approximately 460. The Committee allocated an acceptable daily intake (ADI) "not specified" for isoamylase from the production strain *P. amyloderamosa*, used in the applications specified and in accordance with good manufacturing practice.

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PHOSPHOLIPASE A1 FROM FUSARIUM VENENATUM *EXPRESSED IN* ASPERGILLUS ORYZAE *(addendum)*

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1. EXPLANATION

Phospholipase A1 (phosphatidylcholine 1-acylhydrolase; EC 3.1.1.32) is an enzyme that acts specifically on the fatty acid in position 1 in phospholipid substrates, resulting in the formation of lysophospholipids and free fatty acids. The phospholipase A1 enzyme preparation under evaluation is produced by submerged fermentation of an *Aspergillus oryzae* production strain carrying a gene encoding phospholipase A1 from *Fusarium venenatum.* Its enzyme activity is expressed in lecitase units (LEU), and it is to be used in the dairy industry to produce modified phospholipids in milk used for the manufacture of cheese. This enzyme preparation was evaluated previously by the Committee at its sixty-fifth meeting (Annex 1, reference *178*). The Committee at that meeting concluded that the information provided on phospholipase A1 was too limited to allow an assessment of its safety and that the results of two adequate studies of genotoxicity (including a test for chromosomal aberration in mammalian cells in vitro) and a study of toxicity in vivo would be needed. Alternatives to toxicity testing in vivo would be the demonstration that no unintended compounds are present in the enzyme preparation or better molecular characterization of the production strain. At its present meeting, the Committee evaluated new studies on the toxicity in vivo and genotoxicity of phospholipase A1 and re-evaluated the dietary exposure.

2. BIOLOGICAL DATA

2.1 Toxicological studies

The toxicological studies were performed with a phospholipase A1 liquid enzyme concentrate (LEC) (batch PPW 23436; dry matter content, 6.8% weight per weight [w/w]; total organic solids [TOS] content, 5.6% w/w; specific gravity, 1.027 g/ml), omitting stabilization, formulation and standardization.

2.1.1 Short-term studies of toxicity

Groups of 10 male and 10 female SPF Sprague-Dawley rats (aged 5–6 weeks) were given phospholipase A1 (batch PPW 23436) at a dose equivalent to 0, 57.5, 190 or 575 mg TOS/kg bw by gavage (in tap water), daily for 13 weeks. The study was performed according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 408 (1998) and was certified for compliance with Good Laboratory Practice (GLP) and quality assurance (QA). The animals were checked daily for signs of ill health, behavioural changes, morbidity and mortality. Detailed clinical observations were performed weekly, outside the cage. Body weight and food intake were measured once a week. Ophthalmoscopy was performed on all animals at the start of the experiment and on control and high-dose animals before termination. All animals were tested for their reactivity to different types of stimuli, grip strength and motor activity (open field test) on one occasion during the last 2 weeks of treatment. At termination of treatment, blood samples were taken for haematology and clinical chemistry determinations, and urine was collected for urinalysis. At necropsy, animals were weighed and examined externally. Absolute and relative (to body weight) weights of 11 organs were determined. All animals were examined macroscopically, and 35 organs and tissues from all animals in the control group and at the highest dose were examined microscopically. Microscopy was also performed on all organs and tissues from animals dying or sacrificed during the experiment and on all gross lesions from all animals.

No treatment-related effects were seen on mortality, clinical signs, ophthalmoscopy, sensory reactivity, grip strength, body weights or food consumption. Treated animals showed a few small, but statistically significant, changes in some open field and haematological parameters (female rats) and in some clinical chemistry and urinalysis parameters (male and female rats). None of these changes was considered treatment related, since the values for all changed parameters were well within historical control ranges for these parameters, and most were observed in one sex only, without a dose–response relationship. There were no treatment-related findings on macroscopic or microscopic examination. The only finding on organ weights was a small increase in absolute (not statistically significant) and relative heart weights in females at the highest dose. Given the absence of pathological changes in relevant organs and that values were well within historical control ranges, this finding was not considered to be toxicologically relevant. It can be concluded that the no-observed-effect level (NOEL) is 575 mg TOS/kg bw per day, the highest dose tested in this study (Salanti, 2004).

2.1.2 Genotoxicity

The results of two studies of genotoxicity with phospholipase A1 (batch PPW 23436) in vitro are summarized in Table 1. The first study followed OECD Test Guideline 471 (1997), and the second, OECD Test Guideline 473 (1997). Both studies were certified for compliance with GLP and QA.

Table 1. Genotoxicity of phospholipase A1 in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrApKM101	156–5000 µg/ml, ±S9	Negative[a]	Pedersen (2005)
	S. typhimurium TA100	78–2500 µg/ml, +S9		
	S. typhimurium TA1537	15.6–500 µg/ml, +S9		
Chromosomal aberration	Human lymphocytes	1st experiment: 2812, 3750 or 5000 µg/ml, ±S9 2nd experiment: 3200, 4000 or 5000 µg/ml, –S9; 2560, 4000 or 5000 µg/ml, +S9	Negative[b]	Whitwell (2004)

S9, 9000 × *g* supernatant from rat liver.

[a] By the "treat-and-plate" method (to avoid problems owing to the presence of free amino acids such as histidine and tryptophan in the phospholipase preparation). For the *Salmonella* strains, cell viability was reduced with concurrent reductions in the number of revertants. This was most notable with S9 in strains TA100, TA1535 and TA1537, where viability was less than 20% of control at the higher concentrations tested. When strains TA100 and TA1537 were tested at lower concentrations with S9, reduced cell viability and concurrent reductions in the number of revertants were still observed, but a viability of less than 20% of control was no longer found, except for TA1537 at 500 µg/ml.

[b] With and without metabolic activation from S9. In the first experiment, the cell cultures were treated for 3 h without and with S9 and were harvested 17 h later. The highest tested concentration induced 3% and 15% mitotic inhibition in the absence and presence of S9, respectively. In the second experiment, the cells were exposed continuously for 20 h without S9 and then harvested. With S9, the cells were treated for 3 h and harvested 17 h later. The highest tested concentration induced 33% and 45% mitotic inhibition in the absence and presence of S9, respectively.

3. DIETARY EXPOSURE

The phospholipase A1 enzyme preparation is used in the production of cheese, where it is added to milk prior to coagulation to modify the phospholipids in

milk. After coagulation of the cheese, most of the enzyme is drained off with the whey. Although only a minor portion of the enzyme remains in the cheese, and this portion is inactive owing to lack of substrate, the actual levels of the enzyme (active or inactive) in cheese are not known. A worst-case scenario for human dietary exposure can be estimated on the basis of the recommended use levels and the assumption that all TOS originating from the enzyme preparation are carried over into the cheese. To elaborate this scenario, it is assumed that:

- all cheese is produced from milk processes using the phospholipase A1 enzyme preparation at the highest recommended use level;
- a maximum concentration of 17.5 g of enzyme preparation (or 35 000 LEU) is applied in processing 100 litres of milk;
- 100 litres of milk typically give 10 kg of cheese;
- the TOS content of the enzyme preparation is 2%;
- all TOS are carried over into the cheese.

The resulting maximum concentration of enzyme would correspond to 0.035 mg TOS/g of cheese.

Regarding the consumption of cheese, it is assumed that all cheese from cow's milk reported in the Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme (GEMS/Food) Consumption Cluster Diets was processed using phospholipase A1 (Table 2). Such a scenario would result in a maximum consumption of cheese of 44 g/day (cluster E), corresponding to a dietary exposure to phospholipase of 1.5 mg TOS/day or 0.03 mg TOS/kg bw per day, if a body weight of 60 kg is assumed.

Table 2. Consumption of cheese from the 13 GEMS/Food Consumption Cluster Diets

	Consumption (g/person per day) from GEMS/Food Consumption Cluster Diets												
Commodity	A	B	C	D	E	F	G	H	I	J	K	L	M
Cheese (skim cow's milk)	1	1	2	3	9	1	0	10	3	0	0	3	3
Cheese (whole cow's milk)	0	22	4	3	35	33	0	3	1	0	2	2	33
Total	**1**	**23**	**6**	**6**	**44**	**34**	**0**	**13**	**4**	**0**	**2**	**5**	**36**

In its submission, the sponsor quoted several references for the average consumption of cheese in the United Kingdom (17 g/person per day), Denmark (40 g/person per day) and the United States of America (USA) (15 g/person per day), which are consistent with the estimate based on the 13 GEMS/Food Consumption Cluster Diets.

4. COMMENTS

4.1 Genetic modification / Chemical and technical considerations

The Committee at its sixty-fifth meeting (Annex 1, reference *178*) concluded that the host organism *A. oryzae* is not pathogenic and has a long history of use in food and that the production strain for phospholipase A1, *A. oryzae* PFJo142, constitutes a safe strain lineage. It also concluded that the phospholipase A1 enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing, prepared by the Committee at its fifty-seventh meeting (Annex 1, reference *154*), and that the enzyme preparation is free from the production organism and recombinant deoxyribonucleic acid (DNA).

4.2 Toxicological data

Toxicological studies were performed with a phospholipase A1 LEC. The Committee noted that the materials added to the phospholipase A1 LEC for stabilization, formulation and standardization (e.g. glycerol, sucrose, sodium benzoate and potassium sorbate) either have been evaluated previously by the Committee or are common food constituents and do not raise safety concerns.

In a 13-week study of toxicity in rats, no significant treatment-related effects were seen when the LEC was administered by oral gavage at doses up to and including 575 mg TOS/kg bw per day. Therefore, 575 mg TOS/kg bw per day, the highest dose tested, was taken to be the NOEL. The LEC was not mutagenic in an assay for mutagenicity in bacteria in vitro and was not clastogenic in an assay for chromosomal aberrations in mammalian cells in vitro.

4.3 Assessment of dietary exposure

Based on a maximum mean daily consumption of 44 g of cheese (GEMS/Food Consumption Cluster Diet E) by a 60-kg adult and on the assumptions that the enzyme is used at the maximum recommended use level of 350 LEU/l milk and that all TOS originating from the enzyme preparation remain in the cheese, the dietary exposure would be 0.03 mg TOS/kg bw per day.

5. EVALUATION

Comparing the conservative exposure estimate with the NOEL from the 13-week study of oral toxicity, the margin of safety is >19 000. The Committee allocated an acceptable daily intake (ADI) “not specified” for phospholipase A1 from this recombinant strain of *A. oryzae*, used in the applications specified and in accordance with good manufacturing practice.

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SODIUM IRON(III) ETHYLENEDIAMINETETRAACETIC ACID (SODIUM IRON EDTA) (addendum)

First draft prepared by

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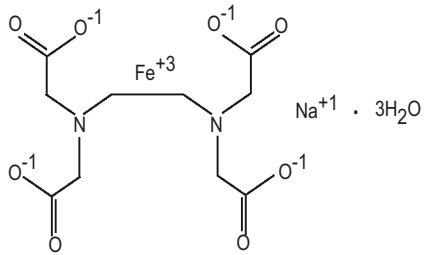
1. EXPLANATION

At the request of the Codex Committee on Food Additives and Contaminants at its thirty-eighth session (Codex Alimentarius Commission, 2006), the Committee re-evaluated the safety of sodium iron(III) ethylenediaminetetraacetic acid trihydrate (in short, sodium iron EDTA) as to its use for iron fortification.

Sodium iron EDTA exists in the trihydrate form, as represented in Figure 1.

Sodium iron EDTA was evaluated previously by the Committee. At its forty-first meeting (Annex 1, reference *107*), the Committee was asked to provide an assessment of the safety of sodium iron EDTA for its use as a dietary supplement in supervised food fortification programmes in populations in which iron deficiency anaemia is endemic. The Committee at that meeting was informed that use of iron

Figure 1. Chemical structure of sodium iron EDTA



in this form would be restricted to this specific application and would be supervised. It was provisionally concluded that sodium iron EDTA that met the tentative specifications prepared at the meeting would not present a safety concern when used in such supervised food fortification programmes. The Committee requested the results of additional studies to assess the site of deposition of iron administered in this form and to assess the metabolic fate of sodium iron EDTA after long-term administration. The Committee emphasized that its evaluation applied only to the use of sodium iron EDTA as a dietary supplement to be used under supervision and expressed its concern about the potential for overfortification of food because of the enhanced bioavailability of iron in this form.

At its fifty-third meeting (Annex 1, reference *143*), the Committee reviewed several studies that were submitted in response to the Committee's request. It was concluded that there was no evidence that administration of iron in the form of sodium iron EDTA would result in greater uptake of iron than that from an equivalent dietary concentration of iron(II) sulfate once the nutritional requirement for iron is satisfied. It was also noted that short-term studies in rats and humans have shown no adverse effects of dietary intake of sodium iron EDTA on the balance of other minerals. The Committee therefore concluded that sodium iron EDTA could be considered safe for use in supervised food fortification programmes, when public health officials had determined the need for iron supplementation of the diet of a population. Such programmes would provide daily intakes of iron and EDTA of approximately 0.2 and 1 mg/kg body weight (bw), respectively, which was considered compatible with previous evaluations by the Committee of both iron and EDTA. For iron, the Committee at its twenty-seventh meeting (Annex 1, reference *62*) had allocated a provisional maximum tolerable daily intake (PMTDI) of 0.8 mg/kg bw to iron from all sources, except for iron oxides and hydrated iron oxides used as food colouring agents and iron supplements taken during pregnancy and lactation or for specific clinical requirements. For EDTA, the Committee at its seventeenth meeting (Annex 1, reference *32*) had established an acceptable daily intake (ADI) of 0–2.5 mg/kg bw for the calcium disodium and disodium salts of EDTA ($CaNa_2EDTA$ and Na_2EDTA). This ADI was expressed as (anhydrous) $CaNa_2EDTA$ and is equivalent to 1.9 mg/kg bw when expressed as EDTA.

The Committee in these previous evaluations thus gave an opinion only on the specific application of sodium iron EDTA in supervised food fortification programmes providing approximately 0.2 mg iron/kg bw per day. This opinion has been perceived as being restrictive to the use of sodium iron EDTA as a source of iron for food fortification (i.e. preventing it from being used at levels higher than 0.2 mg iron/kg bw per day and other than "under supervision only"). The Committee at its present meeting was therefore asked to re-evaluate the safety of sodium iron EDTA for iron fortification. The Committee reviewed data on the safety of sodium iron EDTA that became available after the fifty-third meeting (presented in this addendum) and considered these data together with the data already presented in the previous monographs on sodium iron EDTA.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Rats

Yeung et al. (2004) conducted an iron absorption study to compare the down-regulation of iron absorption from iron(II) sulfate and sodium iron EDTA in rats. Two groups of nine male weanling Sprague-Dawley rats were fed a basal diet containing 35 mg iron (as iron(II) sulfate)/kg for a period of 29 days, while two additional groups were fed a diet containing 30 g elemental iron/kg to induce iron loading over the same period. On day 30, one group of rats fed the basal diet and one group fed the high-iron diet were administered diets radiolabelled with ^{59}Fe (35 mg/kg) from either radiolabelled iron(II) sulfate or sodium iron EDTA, ad libitum for 3 h, followed by whole-body counting. The rats were then fed their respective unlabelled diets containing either iron(II) sulfate or sodium iron EDTA for another 10 days, with whole-body counting at 24-h intervals. Rats were killed on day 41, samples of blood, liver, spleen and kidneys were taken and the three organs were analysed for non-haem iron concentration.

There was no significant difference in concentration of haemoglobin between the groups. Rats fed the high-iron diet had significantly higher concentrations of non-haem iron in liver (9-fold), spleen (4-fold) and kidney (2-fold) compared with those fed the basal diet, indicating elevated iron status. Among rats fed the basal diets, iron retention and absorption were significantly greater in rats fed iron(II) sulfate (64.7%) than in those fed sodium iron EDTA (49.4%). However, in iron-loaded rats, there was no significant difference in absorption between the two iron compounds (12.8% and 10.2%, respectively). In these rats, iron absorption from iron(II) sulfate and sodium iron EDTA was significantly decreased as compared with rats fed the basal diets, with the extent of down-regulation (approximately 80%) comparable between the two iron compounds. This suggests that sodium iron EDTA is no more likely than iron(II) sulfate to exacerbate iron overload when body iron stores are adequate (Yeung et al., 2004).

To study absorption, distribution and excretion of iron from sodium iron EDTA and iron(II) sulfate, 24 male weanling Sprague-Dawley rats were first fed

semipurified diets fortified with 35 mg iron (as iron(II) sulfate)/kg diet for 7 days. After this acclimatization period, the rats were divided into four groups of six rats and fasted overnight. Then, on day 1 of the study, two groups of rats received 2 g test meal containing 70 µg ^{59}Fe coming from either radiolabelled iron(II) sulfate or sodium iron EDTA. The remaining two groups received a subcutaneous injection with 42 µg ^{59}Fe from either radiolabelled iron(II) sulfate or sodium iron EDTA; it was assumed that 100% of this dose is absorbed, making it equivalent to the oral dose, for which it was assumed that there was 60% absorption. ^{59}Fe retention was measured by whole-body counting within 3 h of feeding or injection. Rats subsequently continued on unlabelled diets fortified with the respective iron compounds (providing 35 mg iron/kg) for an additional 10 days, during which 24-h urine samples were collected and analysed for radioactivity. On day 12, rats were killed, and samples of blood, heart, liver, spleen, kidneys, femur bone and muscle around the femur were taken and immediately counted in a whole-body counter, after which the organs were analysed for non-haem iron concentration (Zhu et al., 2006).

Iron excretion was highly increased in the subcutaneous sodium iron EDTA group (77% within 24 h) compared with all other groups and was higher in the oral sodium iron EDTA group than in the oral and subcutaneous iron(II) sulfate groups (1.4%, 0.8% and 0.5% within 24 h, respectively). The cumulative excretion over 10 days in these groups was approximately 85%, 5.8%, 4% and 3.7%, respectively. The absorption and tissue distribution of iron from orally administered iron(II) sulfate or sodium iron EDTA were similar, the absorption being 57.7% and 53.4%, respectively. Following subcutaneous injection, retention and distribution were distinctively different between the two iron compounds, with much higher retention for iron(II) sulfate (91.3%) than for sodium iron EDTA (23.2%). The high excretion found for subcutaneous sodium iron EDTA and the similar patterns found for sodium iron EDTA and iron(II) sulfate after oral administration indicate that iron has to dissociate from the EDTA complex prior to or during uptake by the enterocytes, after which it is regulated similarly to iron from iron(II) sulfate. In all four groups, most radioactivity was retained in blood. Tissue radioactivity levels in the subcutaneous sodium iron EDTA group were significantly lower than those in all other groups, with the exception of the kidney. The authors hypothesized that this could be due to accumulation of ^{59}Fe EDTA in the collecting ducts prior to excretion or, alternatively, dissociation of ^{59}Fe from the complex followed by transport into the interstitial fluid of the kidney. In liver and spleen, but not in heart, kidney, bone and muscle, non-haem iron content was significantly lower (by 56.8% and 28.4%, respectively) in rats consuming the sodium iron EDTA diet as compared with rats fed iron(II) sulfate. Since concentrations of haemoglobin were similar in all groups, an explanation may be that some of the dissociated EDTA is absorbed separately and causes mobilization and redistribution of iron in the body, possibly to the kidneys (Zhu et al., 2006).

2.1.2 Humans

(a) Effect of sodium iron EDTA on the absorption and bioavailability of iron

To examine the regulation of iron absorption in relation to iron status, iron absorption from a whole diet containing a highly bioavailable form of iron was measured for 5 days in 31 healthy men (aged 20–59 years), 12 of whom were blood donors. The non-haem iron and haem iron in this whole diet (i.e. four meals plus two fruit snacks per day) were radiolabelled with ^{59}Fe and ^{55}Fe, respectively. None of the blood donors were anaemic, had signs of iron deficiency erythropoiesis or had given blood during the previous 2 months. However, concentrations of serum ferritin were significantly lower in blood donors (iron-depleted state) than in non-donors (iron-repleted state). The absorptions of haem iron, non-haem iron and total iron were significantly higher in blood donors than in non-donors. The total basal iron requirement (based on total iron losses) was not different between blood donors and non-donors, and in non-donors it did not differ from the total amount of iron absorbed, indicating iron balance. An inverse relationship was noted between total iron absorption and concentration of serum ferritin up to 60–70 µg/l, and this relation was stated to be similar to that in women given the same diet in another study. In iron-repleted states (i.e. serum ferritin >70 µg/l), there was no relation with absorption, as absorption decreased to a level just sufficient to cover basal iron losses, implying that at serum ferritin >70 µg/l no further accumulation of iron in iron body stores will occur by absorption of iron from the diet. Both haem iron absorption and non-haem iron absorption were influenced by iron status: in a state of iron depletion (i.e. at serum ferritin of 10 µg/l), absorption of the two kinds of iron was the same, but as serum ferritin increased, the absorption of both haem and non-haem iron decreased, with the absorption of non-haem iron decreasing more than that of haem iron. This suggests that haem iron absorption may be responsible for a greater part of the total iron absorption in iron-repleted subjects compared with iron-depleted subjects. Comparison of haem and non-haem iron absorption revealed that there was a steep decrease in haem iron absorption with decreasing non-haem iron absorption, indicating that haem iron absorption is effectively controlled. It was stated that the body regulates iron absorption from fortified foods in the same way as dietary non-haem iron (Hallberg et al., 1997).

Several studies with humans were conducted to assess the absorption and bioavailability of iron from meals fortified with sodium iron EDTA or other common forms of iron fortification. A few studies were performed to investigate whether addition of disodium EDTA to other iron fortificants improved iron absorption. Summaries of a number of these studies published after the fifty-third meeting of the Committee are presented below.

The bioavailability of iron from meals based on corn tortillas and black bean paste fortified with iron(II) fumarate with and without disodium EDTA, iron(II) sulfate or sodium iron EDTA (as derived from mixing iron(III) chloride with disodium EDTA in a 1:1 molar ratio of iron to disodium EDTA) was measured in a crossover study with 33 healthy Guatemalan girls (aged 12–13 years and weighing maximally 45 kg) via a stable isotope technique based on erythrocyte incorporation. The test

meals contained 2.2 mg native iron (unlabelled) and 2 mg added radiolabelled iron (^{57}Fe) and had a high content of phytic acid (427 mg), a potent inhibitor of iron absorption. After the girls fasted overnight, the meals were given as breakfast and as lunch on 2 consecutive days, with the alternate test meals administered 14 days later according to the same protocol. This protocol resulted in a level of 4 mg added iron per day, equivalent to approximately 0.1 mg added iron/kg bw per day. Blood samples were taken pretest and 2 weeks after each treatment and analysed for iron status indices (haemoglobin and plasma ferritin) and for incorporation of ^{57}Fe in red blood cells (Davidsson et al., 2002).

None of the girls had a concentration of haemoglobin below 120 g/l. Their concentrations of plasma ferritin ranged from 5 to 48 µg/l, with a mean of 22 µg/l. The iron bioavailability from meals fortified with iron(II) fumarate was 5.5–6.2% and was not improved by the addition of disodium EDTA (5.8–6.7%) at molar ratios of 1:1 relative to total iron (study 1, 11 girls) or to fortification iron (study 2, 11 girls). In study 3 (11 girls), the iron bioavailability from meals fortified with sodium iron EDTA (9.0%) was significantly increased compared with that from meals fortified with iron(II) sulfate (5.5%), indicating that fortification with sodium iron EDTA is more effective than fortification with iron(II) sulfate in staple foods with low iron bioavailability.

Following the same study design, 11 healthy infants (5 boys and 6 girls, 18–27 weeks old and weighing 5.5–8 kg) received an infant cereal (based on wheat flour and soy flour) fortified with 2 mg ^{58}Fe from either radiolabelled sodium iron EDTA or iron(II) sulfate, the latter plus added ascorbic acid (to optimize conditions for absorption) at a molar ratio of 1.6:1 relative to iron. The infant cereal (two servings of 20 g/day) was fed to the infants after an overnight fast or at least 3 h after intake of an infant formula on day 1 of each study, resulting in 4 mg added iron per day (equivalent to 0.5–0.7 mg added iron/kg bw per day). The background level of iron in the cereal was 2 mg/100 g, and it had a phytic acid content of 0.41 g/100 g (Davidsson et al., 2005).

Two infants were anaemic (haemoglobin <110 g/l), and one infant had a low concentration of plasma ferritin (<12 µg/l). Iron bioavailability (as measured by erythrocyte incorporation of ^{58}Fe) from the high-phytate, cereal-based complementary food fortified with sodium iron EDTA (3.7%) was not significantly different from that from the cereal fortified with iron(II) sulfate plus ascorbic acid (4.9%). Under these optimal conditions, both iron compounds were equally efficient in providing bioavailable iron from an inhibitory meal. In an earlier study of Peruvian children (aged 6–7 years) by the same authors, the addition of ascorbic acid (molar ratio to iron 0.6:1) was equally efficient to the addition of disodium EDTA (molar ratio 0.7:1) in enhancing iron bioavailability from a cereal-based school breakfast meal fortified with 14 mg of iron as iron(II) sulfate. Whereas additional ascorbic acid (molar ratio 1.6:1) increased absorption significantly in that study, increasing the molar ratio of disodium EDTA to iron from 0.3:1 to 1:1 had no effect on absorption (Davidsson et al., 2001).

The absorption of iron was studied in nine experiments in a total of 84 men and women (aged 18–40 years, 15 of whom were iron deficient; 7–10 per study)

consuming different cereal foods fortified with iron(II) sulfate, iron(II) fumarate or sodium iron EDTA (at a level of 5 mg added iron) or with iron(II) sulfate or iron(III) pyrophosphate in combination with different concentrations of disodium EDTA (at a level of 4 mg added iron). The absorption of iron from wheat-based, wheat–soybean-based and quinoa-based infant cereals fortified with iron(II) sulfate or iron(II) fumarate ranged from 0.6% to 2.2%, with no significant difference between the two fortificants (absorption ratio 0.9–1.3). Iron absorption from iron(II) sulfate–fortified bread rolls was 1% when the rolls were made from high-extraction wheat flour and 5.7% when they were made from low-extraction wheat flour. When sodium iron EDTA was used as fortificant, the absorption of iron from all three infant cereals and the two types of bread rolls was 2.0–3.9 times greater than when the same product was fortified with iron(II) sulfate. Sodium iron EDTA, however, did not completely reverse the phytate-inhibiting effect on iron absorption, given that the absorption of iron was lower in products with high phytate content (wheat–soybean-based and quinoa-based infant cereals and bread rolls from high-extraction wheat flour; 2.8%, 1.7% and 3.9%, respectively) than in products with low amounts of phytate (wheat-based infant cereal; 5.2%) or even no phytate present (bread rolls from low-extraction wheat flour; 11.5%). The inhibitory effect of polyphenols from tea was also not completely reversed, as the administration of tea with bread rolls from low-extraction wheat flour significantly decreased iron absorption from both sodium iron EDTA and iron(II) sulfate (from 11.5% to 1.9% and from 5.7% to 1.0%, respectively). Increasing the fortification level from 5 to 15 mg in the wheat–soybean-based infant cereal decreased iron absorption from both sodium iron EDTA (from 3.3% to 1.9%) and iron(II) sulfate (from 0.9% to 0.7%), but iron absorption from sodium iron EDTA was still significantly greater than that from iron(II) sulfate. When disodium EDTA up to a 1:1 molar ratio (EDTA to iron) was added to iron(II) sulfate–fortified wheat-based and wheat–soybean-based infant cereal, iron absorption from the wheat cereal increased from 1.0% to a maximum of 5.7% at a molar ratio of 0.67:1. An increase, but smaller, was also observed for the wheat–soybean cereal (from 0.7% to a maximum of 2.9% at a molar ratio of 1:1). When directly comparing iron(II) sulfate– and iron(III) pyrophosphate–fortified wheat-based infant cereal, iron absorption from the latter was lower than, but not significantly different from, that of iron(II) sulfate. However, whereas addition of disodium EDTA (at a 1:1 molar ratio) significantly increased iron absorption from iron(II) sulfate (from 1.8% to 5.9%), it did not do so for iron(III) pyrophosphate (marginal increase from 0.3% to 0.4%) (Hurrell et al., 2000).

In a study comparing iron bioavailability from different fortificants, two groups of 13 healthy adult men (aged 15–50 years) and women (some of whom were postmenopausal; exact number not given) were provided control meals from precooked corn or from wheat flour with a basal iron content of 1.5 mg and 1.6 mg, respectively, or these meals fortified with sodium iron EDTA, iron(II) sulfate or iron(II) bisglycinate (Ferrochel), providing 3 mg added iron. Following consumption of meals prepared with either corn or wheat flour, iron absorption from foods fortified with iron(II) bisglycinate (8.4% and 10.8%, respectively) or sodium iron EDTA (10.5% and 14.9%, respectively) was significantly greater than that obtained from unfortified (3.2% and 3.0%, respectively) or iron(II) sulfate–fortified foods (4.7% and 5.3%, respectively). In both groups, 4 of 13 subjects were iron deficient. Their iron

absorption was also reported to be significantly greater from foods fortified with iron(II) bisglycinate (12–13%) or sodium iron EDTA (14–15%) than from iron(II) sulfate–fortified foods (6–7%) (Layrisse et al., 2000).

A more efficient absorption of iron from sodium iron EDTA–fortified food as compared with iron(II) sulfate–fortified food was also observed in a study with 14 non-pregnant women (aged 19–42 years, all non-anaemic, but 4 women with a concentration of serum ferritin <12 µg/l) given porridge prepared from maize dough with either a low or a normal phytate content (ratio phytate:iron, 6.8:1 and 16:1, respectively). The porridges contained 3.4 mg iron per serving, to which an extra 1 mg iron was added from sodium iron EDTA or iron(II) sulfate. Absorption of iron from sodium iron EDTA–fortified porridge (5.4% and 5.7% for low and normal phytate contents, respectively) was significantly higher than that from iron(II) sulfate–fortified porridge (1.9% and 1.7% for low and normal phytate contents, respectively). Phytate content had no significant effect on iron absorption from porridges fortified with either sodium iron EDTA or iron(II) sulfate (Mendoza et al., 2001).

Groups of healthy, non-pregnant women (aged 31–50 years) received tortillas that were prepared from corn masa, which contained 15 mg native iron/kg flour. These tortillas were given unfortified, either with or without disodium EDTA, or fortified at 30 mg/kg flour with added iron from iron(II) fumarate (with or without disodium EDTA), iron(II) bisglycinate (Ferrochel) or sodium iron EDTA. Absorption of iron from meals that were not supplemented with disodium EDTA (0.69%, 0.87% and 1.27% for native iron, iron(II) fumarate and iron(II) bisglycinate, respectively) was significantly decreased in comparison with absorption from meals supplemented with disodium EDTA (3.19% and 2.98% for native iron and iron(II) fumarate, respectively). The highest absorption of iron was noted following consumption of the meal fortified with sodium iron EDTA (5.3%), but this was not statistically significantly different from the absorption of iron from meals supplemented with disodium EDTA (Walter et al., 2003).

In order to study the iron absorption from sodium iron EDTA–fortified fish sauce and soy sauce, groups of 10 women (aged 19–29 years, weighing maximally 60 kg, 2 with iron deficiency anaemia and 16 with iron deficiency) were given these fortified sauces added to a meal of rice and vegetable purée or rice only in a crossover study design. The meals were given after an overnight fast, on 2 consecutive days. The total iron content of the test meals varied between 5.1 and 5.9 mg, 5 mg of which came from fortification. The phytic acid content of the test meals was relatively low (25 mg and 27 mg for the meals with fish sauce and soy sauce, respectively). Iron absorption from sodium iron EDTA–fortified fish sauce and soy sauce was relatively high when added to rice and vegetable purée (3.3% and 6.1%, respectively) and was not significantly different from iron absorption from identical meals with iron(II) sulfate–fortified fish sauce and soy sauce (3.1% and 5.6%, respectively). Hence, with the tested meals that were moderately inhibitory as to phytate content, there was no enhancing effect of sodium iron EDTA on absorption relative to iron(II) sulfate. When compared directly, no significant difference in iron absorption was observed for sodium iron EDTA–fortified fish sauce (6.7%) or sodium iron EDTA–fortified soy sauce (7.9%) added to rice only (Fidler et al., 2003).

(b) Effect of EDTA compounds on the absorption of other elements

In a study conducted with 11 infants, as well as bioavailability of iron (see above), the effect of fortification with either sodium iron EDTA or iron(II) sulfate (plus ascorbic acid) on the absorption of zinc, copper, calcium and magnesium was investigated. Of the two servings of infant cereal administered to the infants, serving 1 also contained stable isotope–labelled zinc (888 µg as $^{70}ZnCl_2$) and calcium (4.0 mg as $^{44}CaCl_2$) in addition to labelled iron, while serving 2 contained stable isotope–labelled copper (1.0 mg as $^{65}CuCl_2$) and magnesium (5.0 mg as $^{25}MgCl_2$) as well as equilibrated amounts of normal isotopic iron, zinc and calcium. Following ingestion of the meals, faecal material was collected for 72 h. During the 72-h periods, infants were fed a standardized diet consisting of low-iron infant formula and two servings per day of 20 g wheat–soy infant cereal containing added food-grade sodium iron EDTA or iron(II) sulfate at a concentration of 10 mg iron/100 g. The investigators did not find significant differences in the apparent absorption of zinc (20.5% vs 21.1%), copper (8.9% vs 11.1%), calcium (50.6% vs 50.0%) or magnesium (47.9% vs 49.6%) between test meals fortified with sodium iron EDTA and those fortified with iron(II) sulfate plus ascorbic acid, respectively (Davidsson et al., 2005).

In order to study the bioavailability of iron and zinc from a locally prepared rice flour–based meal, 53 Sri Lankan children aged 7–10 years were randomly divided into four groups. The rice flour was fortified with iron (60 mg/kg, as iron(II) sulfate) and folate (2 mg/kg), and in two groups also with zinc (60 mg/kg, as zinc oxide), and was given with and without disodium EDTA (385.08 mg/kg, to provide a ratio of 1:1 to iron and 1:0.7 to zinc). Subjects consumed the fortified diets for a period of 14 days prior to an absorption trial, in which subjects consumed their respective test diets, which were prepared with radiolabelled iron and/or zinc. Baseline measurements indicated that 38% of subjects were anaemic, 8% had low serum ferritin, 36% were deficient in folate and 15% were deficient in zinc. In the 48 subjects completing the trial, the iron, zinc and folate status was generally improved, the improvement being the smallest when no disodium EDTA or zinc was added. In subjects consuming meals with disodium EDTA, the absorption of iron was significantly increased as compared with those not receiving disodium EDTA. Additional zinc did not result in a significant difference in iron absorption. In the two groups receiving the zinc-fortified rice flour, absorption of zinc was higher in the group with disodium EDTA (13.5%) than in the group without disodium EDTA (8.8%) (Hettiarachchi et al., 2004).

In a study comparing iron and zinc absorption from a standard and novel dry food supplement designed for preschool children, the standard food supplement contained a fortification mixture of 10 mg iron as iron(II) sulfate, 10 mg zinc as zinc sulfate and 50 mg ascorbic acid per 90 g, whereas the novel food supplement contained 10 mg iron as sodium iron EDTA, 10 mg zinc as zinc methionine, 100 mg ascorbic acid and 1 g citric acid per 90 g. Additionally, both types of food products were prepared with a low (192 mg; background level) or high (192 mg background level + 200 mg added as dicalcium phosphate) calcium content. Thirteen healthy, non-pregnant women (aged 20–31 years) were provided with one of the four different food products prepared as porridge (i.e. standard food supplement with a low or high calcium content, or novel food supplement with a low or high calcium

content) at 7-day intervals. The absorption of iron from the novel food product containing sodium iron EDTA was significantly higher (1.7 times) than that from the standard food product containing iron(II) sulfate. Dietary calcium levels had no significant effect on iron absorption from the standard (1.5% and 1.6% for low and high calcium content, respectively) and novel food product (2.7% and 2.5% for low and high calcium content, respectively). When no extra calcium was added, zinc absorption from the EDTA-containing food product (8.2%) was somewhat higher than that from the standard food product (6.7%). At high dietary calcium, there was no difference in zinc absorption from the two products (both 6.6%). For the novel food product, however, higher dietary calcium was marginally associated with lower zinc absorption (6.6% vs 8.2%) (Mendoza et al., 2004).

2.2 Toxicological studies

2.2.1 Acute toxicity

The acute oral toxicity of sodium iron EDTA was examined in one study in young male Sprague-Dawley rats (4–8 per dose) given the substance at a single dose of 5, 10, 12.5, 15, 20, 30 or 40 g/kg bw by gavage. The LD_{50} was 10 g/kg bw (Whittaker et al., 2002).

2.2.2 Short-term studies of toxicity

No new information was available.

2.2.3 Long-term studies of toxicity and carcinogenicity

No new information was available.

2.2.4 Genotoxicity

The results of three studies of genotoxicity with sodium iron EDTA in vitro are summarized in Table 1. The first study was a so-called WP2 Mutoxitest, using *Escherichia coli* strains WP2uvrA/pKM101 (designated IC188) and IC203, a derivative of IC188, sensitive in detecting oxidative mutagens (Martínez et al., 2000). The second and third studies (which were already available to the Committee at its fifty-third meeting, but are rectified here) used *Salmonella typhimurium* strains and mouse lymphoma cells, respectively (Dunkel et al., 1999).

2.2.5 Reproductive toxicity

No new information was available.

Table 1. Genotoxicity of sodium iron EDTA in vitro

End-point	Test system	Concentration	Result	Reference
Reverse mutation	*E. coli* IC188 and IC203	Up to 5000 µg/plate	Negative[a]	Martínez et al. (2000)
Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537 and TA1538	Up to 10 000 µg iron/plate	Negative[b]	Dunkel et al. (1999)
Gene mutation	Mouse lymphoma L5178Y TK+/– cells	1.3–325 µg iron/ml, –S9; 0.03–6.5 µg iron/ml, +S9	Positive[c]	Dunkel et al. (1999)

S9, 9000 × *g* supernatant from rat liver.

[a] Without metabolic activation (S9), by the direct plate incorporation method.

[b] With and without metabolic activation (S9), by the direct plate incorporation method. In addition, strain TA102 was tested by the preincubation method.

[c] With and without metabolic activation (S9). Without S9, a positive response was seen only at 325 µg/ml (2.4-fold increase in mutation frequency), which also produced cytotoxicity (relative total growth, 33.5% of control). With S9, positive responses were observed at 1.625, 3.25 and 6.5 µg/ml (2.2-, 2.5- and 2.7-fold increases in mutation frequency, respectively), which also produced cytotoxicity (relative total growth, 53%, 38% and 19% of control, respectively). The Committee noted that other iron(II) or iron(III) salts also showed positive results at the higher (mostly cytotoxic) concentrations tested, whereas disodium EDTA, the non-iron-containing control substance for sodium iron EDTA, tested negative.

2.3 Observations in humans

2.3.1 Sodium iron EDTA–fortified fish sauce

A randomized, double-blind, placebo-controlled study was performed in Viet Nam to test the efficacy of iron-fortified fish sauce in improving the iron status of anaemic women. The women recruited were factory workers who had to be non-pregnant and anaemic (haemoglobin concentration <120 but >80 g/l), and study sites did not have interventions to control iron deficiency anaemia. Two groups of 76 women each (17–49 years of age, mean weight 48 kg) received a meal based on noodles or rice with 10 ml fish sauce containing either 10 mg iron as sodium iron EDTA (iron-fortified group) or no added iron (control group), 6 days/week for 6 months. Concentrations of haemoglobin, serum ferritin (SF) and serum transferrin receptor (TfR) were measured at baseline and after 3 and 6 months, and TfR:SF ratios and total body iron content (using the TfR:SF ratios) were calculated. Fifteen women (12 from the iron-fortified group and 3 controls) did not complete the study, and 1 woman from the control group was excluded because of excessive blood loss (Thuy et al., 2003).

At baseline, the two groups did not differ in age, height, weight, body mass index or dietary intakes of energy and selected foods and nutrients (including iron, which was approximately 9 mg/day in both groups). The prevalence of infections,

as determined by measuring the concentration of C-reactive protein in serum and counting of intestinal parasites, was also not different, nor were there significant differences between the two groups at baseline in the prevalence of anaemia, iron deficiency (i.e. serum ferritin <12 µg/l and/or TfR >8.5 mg/l) or iron deficiency anaemia (i.e. anaemia plus serum ferritin <12 µg/l and/or TfR >8.5 mg/l), or in TfR:SF ratios and total body iron content. After 3 and 6 months, concentrations of haemoglobin and serum ferritin and total body iron content were significantly higher, and concentrations of TfR and TfR:SF ratios were significantly lower in the iron-fortified group than in the control group. By the end of the study, the prevalence of anaemia, iron deficiency and iron deficiency anaemia had significantly decreased in the iron-fortified group (from 100% to 66%, 70% to 32% and 70% to 20%, respectively), but not in the control group.

The effectiveness of iron fortification in controlling iron deficiency in women of childbearing age in Viet Nam was investigated in an 18-month double-blind intervention study with randomization by village. Study sites were selected based on a high prevalence of anaemia (>20%) and absence of interventions to control iron deficiency anaemia. All households in 21 selected villages in two rural communes were supplied with fish sauce that was either unfortified (10 villages) or fortified with sodium iron EDTA at a concentration of 9 mmol (approximately 500 mg) iron/l (11 villages). Sufficient fish sauce was provided to allow a daily consumption of 15 ml fish sauce for every household member for 18 months, equivalent to 7.5 mg iron/person per day. The effect of fortification was assessed in 576 women (288 per group; randomly selected from households with at least one non-pregnant woman aged 16–49 years; mean weight 45 kg) by measuring concentrations of haemoglobin, serum ferritin and TfR at baseline and after 6, 12 and 18 months (t_6, t_{12} and t_{18}, respectively). Owing to technical problems TfR concentrations were considered unreliable and thus not reported. In total, 188 women (89 from the iron-fortified group and 99 controls) were lost during follow-up (Thuy et al., 2005).

At baseline and during the study, the two groups did not differ in age, height, weight, body mass index or dietary intakes of energy and selected foods and nutrients (including non-fortification iron, which was 9–10 mg/day in both groups). Based on results from measurements of serum concentrations of retinol and C-reactive protein and of intestinal parasite counts, it was concluded that the iron deficiency in the investigated population is likely caused primarily by inadequate intake of iron, and not by iron losses due to inflammatory disorders, vitamin A deficiency or infections with gastrointestinal parasites. At baseline, the two groups did not differ significantly in concentrations of haemoglobin and serum ferritin and in prevalence of anaemia (24–25%) and iron deficiency (22%). Fortification resulted in progressively higher concentrations of haemoglobin and serum ferritin when compared with controls, the difference between the two groups being statistically significantly at t_6 (serum ferritin only), t_{12} and t_{18}. During the study, the prevalence of anaemia and iron deficiency decreased in the iron-fortified group (to 8.5% and 4% at t_{18}, respectively), but not in the control group. Compared with the control group, the difference in prevalence was significant at t_{18} for anaemia and at t_6, t_{12} and t_{18} for iron deficiency.

2.3.2 Sodium iron EDTA–fortified soy sauce

An 18-month, randomized, placebo-controlled intervention trial was performed to study the effectiveness of sodium iron EDTA–fortified soy sauce in controlling iron deficiency in a high-risk population in a rural area in China. Households in five villages (7684 residents ≥3 years of age) were supplied with soy sauce that was fortified with sodium iron EDTA at a concentration of 29.6 mg iron/100 ml. Households in four villages (6332 residents ≥3 years of age) served as controls and were supplied with unfortified soy sauce. Sufficient soy sauce was provided monthly to allow a daily consumption of 15 ml (equivalent to 4.4 mg iron) by each family member, for 18 months in total. The evaluation cohort was selected based on school classes (i.e. when selecting students class by class, all their family members ≥3 years of age were also selected), until about one third of all the participants were selected. Nine subgroups were formed, based on age and sex (3–6, 7–18, 19–54 and 55+ years for males, and 3–6, 7–18, 19–30, 31–54 and 55+ years for females). The total number of subjects in each of these subgroups represented approximately one third of the whole population in that age and sex subgroup. Individual food frequency questionnaires were distributed at baseline and at 6, 12 and 18 months (t_0, t_6, t_{12} and t_{18}, respectively). Blood samples were also drawn at these time points and analysed for haemoglobin (in t_0, t_6, t_{12} and t_{18} samples of all age groups), plasma ferritin (in t_0 and t_{12} samples of 7–55+ age groups) and plasma retinol (in selected t_0 and t_{12} samples of 7–55+ age groups) (Chen et al., 2005).

No significant differences in food intake or food preparation methods between the fortified and control groups or between the beginning and the end of the study were observed. The estimated total daily iron intake was high (14.9–20.5 mg/day for children and adolescents, and 22.1–27.5 mg/day for adults) and met or exceeded the recommended dietary allowance (RDA) in all subgroups. However, the bioavailability of the dietary iron is likely low, given that most of it came from cereals and fruits and vegetables. On average, the iron consumed from the fortified soy sauce was 3.8 mg/person per day, based on an average consumption of 16.4 ml/day and an actual measured concentration of 23 mg iron/100 ml soy sauce. (Note: The authors calculated an average of 4.9 mg additional iron/day, based on the average consumption of 16.4 ml/day and the intended concentration of 29.4 mg iron/100 ml.) After 1 year of intervention, the scores for weight-for-age, weight-for-height and height-for-age were higher in the iron-fortified 3–6 years subgroup than in the control 3–6 years subgroup, with no differences observed for all other subgroups. At baseline, the prevalence of hookworm infections (as determined by analysis of stool samples) was very low, and the fortification and control groups did not differ significantly in concentrations of haemoglobin (ranging from 111 to 135 g/l for the different age groups), plasma ferritin (3.1–6.6 µg/l) and plasma retinol (19–40 µg/dl) and in prevalence of anaemia (28–64%). These values indicate that in most subjects, anaemia (i.e. haemoglobin <110–130 g/l, depending on age) was mild, but iron deficiency (i.e. plasma ferritin <12 µg/l) was considerable. Moreover, a large number of subjects appeared to have either clinical or subclinical vitamin A deficiency (i.e. plasma retinol <20 and <30 µg/dl, respectively), especially in the 7–18 years subgroups. The Committee noted that no data on plasma ferritin or

plasma retinol are available for children aged 3–6 years, since no venous blood was drawn from this age group. Concentrations of haemoglobin were significantly higher than the baseline values in each of the fortified subgroups at t_6, t_{12} and t_{18}, with the largest increase in the first 6 months and only little change afterwards. A significant increase in concentration of haemoglobin as compared with baseline values was also observed in the control subgroups at most time points, but this increase was 2–3 times smaller than in the fortified subgroups. Compared with control treatment, fortification resulted in higher concentrations of haemoglobin for all sex and age subgroups at all sampling times, although no statistical significance was reached for men aged 55+ years and girls aged 3–6 years at t_6 and t_{12} and for boys aged 3–6 years at t_6. The prevalence of anaemia was significantly decreased in each of the fortified subgroups at t_6, t_{12} and t_{18} (to less than 50% of the baseline values), with the largest decrease in the first 6 months, a further, smaller decrease at t_{12} and a slight increase at t_{18}. The prevalence of anaemia was decreased in all control subgroups as well (except for children aged 3–6 years, where at t_{18} an overall increase was observed), but the decrease was considerably smaller than in the fortified groups. Compared with control treatment, fortification resulted in a lower prevalence of anaemia for all sex and age subgroups at all sampling times, although for the 3–6 years and 55+ years subgroups, statistical significance was not reached for all time points. After 1 year of intervention, the concentration of plasma ferritin was significantly increased above baseline values in all examined fortified subgroups, but was still below 12 µg/l, except for men aged 19–54 years. All examined control subgroups also showed an increase, but smaller, in concentration of plasma ferritin, except for men and women aged 55+ years, for whom the increase was comparable with that in the 55+ years fortified subgroups. The difference between treated and control groups in concentration of plasma ferritin reached statistical significance for males aged 7–18 and 19–54 years and for females aged 7–18 years. An overall moderate improvement in vitamin A status was observed in both the examined fortified subgroups and the examined control subgroups after 1 year of intervention, the origin of which is unclear.

The effect of sodium iron EDTA–fortified soy sauce on anaemic adolescents (11–17 years of age, concentration of haemoglobin <120 g/l for girls and <120–130 g/l for boys) was investigated in three boarding schools in China. In total, 304 children (155 boys, 149 girls) were enrolled in the fortification intervention study, in which they were given soy sauce soup at lunch, daily for 3 months. This soup contained 5 ml soy sauce that was either unfortified (school 1, 55 boys and 49 girls) or fortified with sodium iron EDTA at a concentration of 1 mg iron/ml (school 2, 39 boys and 63 girls) or 4 mg iron/ml (school 3, 61 boys and 39 girls). Concentrations of free erythrocytic porphyrin, serum iron, serum ferritin, serum transferritin and total iron binding capability were determined at baseline and after 3 months, whereas blood haemoglobin was measured at baseline and after 1, 2 and 3 months (Huo et al., 2002).

The average daily iron intake, as derived from dietary surveys at the three school cafeterias using a 3-day food weighing method, was approximately 17 mg, with no difference between the schools. Although this iron intake is adequate when compared with the RDA, the bioavailability is likely low given that most of the daily

iron intake came from cereals (84%) and vegetables (7.5%). Concentrations of haemoglobin before the intervention were not statistically significantly different between the three groups (mean levels ranging from 115 to 117 g/l). Fortification resulted in progressively higher concentrations of haemoglobin: at the high level (20 mg/day), concentrations of haemoglobin were significantly higher than baseline and control values after 1, 2 and 3 months, whereas at the low level (5 mg/day), this occurred after 2 and 3 months. It was reported that two subjects in the low-level fortification group, one subject in the high-level fortification group and 69.5% of the control group still suffered from anaemia at the end of the study. Concentrations of serum iron and serum ferritin were significantly higher and concentrations of free erythrocytic porphyrin, total iron binding capability and serum transferritin were significantly lower in both fortification groups at the end of the study compared with baseline and control values. Although the increase in haemoglobin and the decreases in free erythrocytic porphyrin, total iron binding capability and serum transferritin were somewhat greater in the high-fortification group compared with the low-fortification group, their concentrations did not differ statistically significantly between both fortification groups at the end of the study. The Committee noted that the reporting of this study was inaccurate and lacking detail. It was stated that of the 4008 eligible students in the three schools, 304 students were diagnosed as anaemic or suffered from iron deficiency anaemia. However, prevalence values of anaemia diagnosed indicated more than 600 eligible students to be anaemic, and mean baseline concentrations of serum ferritin (ranging from 42 to 48 µg/l) do not indicate iron deficiency. Furthermore, although 304 students participated in the study, results were presented for only 240 of them, with no explanation given. It was also stated that the subjects had similar living standards and dietary patterns, but a dietary survey was conducted only before the start of the intervention.

2.3.3 Sodium iron EDTA–fortified whole maize flour

The effect of consumption of whole maize flour fortified with iron as sodium iron EDTA (at 28 or 56 mg iron/kg flour) or electrolytic iron (at 56 mg/kg flour) on iron status was investigated in Kenyan schoolchildren aged 3–8 years. Five hundred and sixteen children from four schools were randomly assigned to one of four groups, receiving porridge made from unfortified whole maize flour or porridge from whole maize flour fortified with high-dose sodium iron EDTA, low-dose sodium iron EDTA or electrolytic iron. The target amount of this high-phytate meal (700 ml of porridge containing 100 g flour for children aged 3–5 years and 1000 ml of porridge containing 150 g flour for children aged 6–8 years) was provided daily to every child in two equally divided portions, 5 days/week for 5 months. These amounts were targeted to provide 18–20% (low-dose iron) and 36–40% (high-dose iron) of the daily iron requirements for children of these age groups. Concentrations of haemoglobin, plasma ferritin and plasma soluble transferrin receptor were determined at baseline and after 5 months. All children received malaria chemotherapy 2 weeks before final blood collection to avoid inflammation-induced effects on iron status indicators (Andang'o et al., 2007).

At baseline, almost half the children (254) had current or recent malaria infection. Two hundred and ninety (56%), 78 (15%) and 54 (11%) children had

anaemia (i.e. haemoglobin <110–115 g/l, depending on age), iron deficiency (i.e. plasma ferritin <12–15 µg/l, depending on age) and iron deficiency anaemia (i.e. concurrent anaemia and iron deficiency), respectively. Several children had α-thalassaemia, scored a positive result for the sickling test and/or had worm infections (mostly hookworm). Concentrations of C-reactive protein, however, showed few children with inflammation (i.e. C-reactive protein >10 mg/l). Five hundred and five children completed the study, and groups assigned to receive porridge fortified with high-dose sodium iron EDTA, low-dose sodium iron EDTA or electrolytic iron or porridge from unfortified flour consumed 92%, 89%, 90% and 93%, respectively, of the total amount given to them during the intervention period. Consumption of high-dose and low-dose sodium iron EDTA–fortified flour dose dependently improved the iron status of children, as indicated by increased concentrations of haemoglobin and plasma ferritin and decreased concentrations of plasma soluble transferrin receptor compared with unfortified flour. In contrast, fortification with electrolytic iron did not affect any of the three iron status indicators. Iron status at baseline modified the effect of sodium iron EDTA: in children with iron deficiency or iron deficiency anaemia, the effect of both high- and low-dose sodium iron EDTA on iron status indicators was greater (dose dependently) than the effect in iron-replete children. Relative to placebo treatment, high-dose sodium iron EDTA reduced the prevalence of anaemia (by 36%), iron deficiency (by 91%) and iron deficiency anaemia (by 89%). Low-dose sodium iron EDTA also reduced iron deficiency (by 70%) but did not change the prevalence of anaemia. Electrolytic iron did not confer protection against any of these disorders; relative to placebo, the prevalence of iron deficiency anaemia was even increased. There was no influence of malaria, α-thalassaemia or sickling test result on the effects of fortification with sodium iron EDTA.

2.3.4 Special considerations

According to the dossier submitted, sodium iron EDTA as a source of iron in fortified foods at levels providing 0.2 mg iron/kg bw per day cannot be effectively used in treating iron deficiency in infants and young children, given the much higher RDA values for iron for these age groups (see Table 2). In order for sodium iron EDTA to cover a greater part of the RDAs, maximum intake levels of iron as sodium iron EDTA have been suggested in the data submission (see Table 2), which are considered sufficient to treat iron deficiency in all age groups in any population consuming high-phytate diets. The Committee noted, however, that these higher levels of fortification with sodium iron EDTA may lead to an intake of EDTA in infants and children up to the age of 13 that is at or exceeds the acceptable level of 1.9 mg/kg bw for EDTA (i.e. the equivalent of the ADI of 0–2.5 mg/kg bw for calcium disodium EDTA and disodium EDTA). The Committee additionally noted that no account was taken of the intake of EDTA from the food additive use of other EDTA compounds, such as, for example, calcium disodium EDTA and disodium EDTA, which amounts to 0.1–0.2 mg/kg bw per day.

Table 2. Suggested maximum intakes of iron as sodium iron EDTA (as compared with RDA values for iron) and corresponding maximum intakes of EDTA

Age	Reference body weight[a] (kg)	RDA for iron[a]		Suggested maximum iron intake (as sodium iron EDTA)		Corresponding maximum intake of EDTA[b] (mg/kg bw per day)
		(mg/day)	(mg/kg bw per day)	(mg/day)	(mg/kg bw per day)	
Infants and children: males and females						
7–12 months	9	11	**1.2**[c]	5	0.56	**2.9**
1–3 years	13	7	0.54	5	0.38	**2.0**
4–8 years	22	10	0.45	8	0.36	**1.9**
9–13 years	40	8	0.20	15	0.38	**1.9**
Adolescents and adults: males						
14–18 years	64	11	0.17	18	0.28	1.5
19–>70 years	76	8	0.11	18	0.24	1.2
Adolescents and adults: females						
14–18 years	57	15	0.26	18	0.32	1.6
19–50 years	61	18	0.30	18	0.30	1.5
51–>70 years	61	8	0.13	18	0.30	1.5
Pregnancy						
14–50 years	[61]	27	[0.44]	18	0.30	1.5
Lactation						
14–18 years	[57]	10	[0.18]	18	0.32	1.6
19–50 years	[61]	9	[0.15]	18	0.30	1.5

[a] Source: Institute of Medicine (2001) (no body weights specified at pregnancy and lactation).

[b] Calculated as (intake iron/molecular $weight_{iron}$) × molecular $weight_{EDTA}$ = (intake iron/56) × 288.

[c] Values in bold are either in excess of the PMTDI of 0.8 mg/kg bw for iron or at or in excess of 1.9 mg/kg bw, the equivalent of the ADI of 0–2.5 mg/kg bw for calcium disodium EDTA and disodium EDTA when expressed as EDTA.

3. COMMENTS

In addition to the studies already evaluated by the Committee at its earlier meetings, several new studies on the biochemical and toxicological aspects and on the efficacy of sodium iron EDTA were submitted.

The new data provided on the biochemical aspects following administration of sodium iron EDTA corroborate the findings from earlier studies, i.e. that

- the iron in sodium iron EDTA dissociates from the chelate and is released into the common non-haem iron pool before absorption;
- only a very small fraction of the sodium iron EDTA complex (less than 1–2%) is absorbed intact and is rapidly and completely excreted via the kidneys in the urine;
- iron from sodium iron EDTA is generally more efficiently absorbed than iron from alternative sources such as iron(II) sulfate, with less influence of inhibitory factors present in the diet (e.g. phytic acid, polyphenols);
- the same enhancing effect on iron absorption can be achieved by adding disodium EDTA to other soluble iron fortificants, such as iron(II) sulfate.

Moreover, it was demonstrated that the human body maintains iron levels through down-regulating systems, which control the amount of iron absorbed and protect against the possibility of iron overload: more iron is absorbed when the body is in a state of iron deficiency (with an inverse relation between absorption and concentration of serum ferritin) and less when the body iron stores are replete. Besides, a 3-fold increase in the level of iron fortification resulted in a decreased percentage of iron absorption from sodium iron EDTA in healthy adults. Also, in iron-replete subjects, non-haem iron absorption was reduced to a larger extent than the absorption of haem iron, whereas absorption of iron during states of iron depletion was comparable for the two different forms of iron. This tight regulation of non-haem iron absorption is of importance, given that iron from sodium iron EDTA will join the non-haem iron pool before absorption. These findings support the conclusion that dietary iron fortification with sodium iron EDTA does not increase the risk for iron accumulation beyond normal physiological requirements in iron-replete individuals. Studies have also provided additional evidence that dietary intake of sodium iron EDTA has no negative influence on the absorption of other minerals, such as zinc.

From the new toxicological studies provided, it can be concluded that sodium iron EDTA is of low acute oral toxicity and that it does not induce gene mutations in bacteria and mammalian cells in vitro, unless tested at high, cytotoxic concentrations.

The results of new intervention studies in populations with a high prevalence of anaemia and iron deficiency in Viet Nam, China and Kenya demonstrated the efficacy of sodium iron EDTA–fortified condiments (soy sauce and fish sauce) and sodium iron EDTA–fortified whole maize flour in reducing iron deficiency and/or iron deficiency anaemia and the prevalence of anaemia.

4. EVALUATION

The Committee considered the new data submitted to be in support of the earlier conclusions by the Committee at its fifty-third meeting that once the nutritional requirement for iron is satisfied, administration of iron in the form of sodium iron EDTA will not result in greater uptake of iron than from other iron fortificants (such as iron(II) sulfate), as a result of down-regulating systems in the body. The

Committee also noted that the new data submitted provide additional evidence that dietary intake of sodium iron EDTA has no adverse effects on the absorption of other minerals, such as zinc.

The Committee concluded that sodium iron EDTA is suitable for use as a source of iron for food fortification to fulfil the nutritional iron requirements, provided that the total intake of iron from all food sources including contaminants does not exceed the PMTDI of 0.8 mg/kg bw (Annex 1, reference *62*). Additionally, the total intake of EDTA should not exceed acceptable levels, also taking into account the intake of EDTA from the food additive use of other EDTA compounds. An ADI of 0–2.5 mg/kg bw was previously established for the calcium disodium and disodium salts of EDTA, equivalent to up to 1.9 mg EDTA/kg bw (Annex 1, reference *32*). A preliminary exposure assessment based on suggested levels of fortification for sodium iron EDTA indicates that the intake of EDTA in infants and children up to the age of 13 already is at or exceeds the upper limit of the ADI for EDTA.

As previously noted for ferrous glycinate (Annex 1, reference *166*), products, including sodium iron EDTA, that are intended to provide a source of additional iron should not be consumed by individuals with any type of iron storage disease, except under medical supervision.

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SAFETY EVALUATIONS OF GROUPS OF RELATED FLAVOURING AGENTS

INTRODUCTION

Eight groups of flavouring agents were evaluated using the Procedure for the Safety Evaluation of Flavouring Agents as outlined in Figure 1 (Annex 1, references *116*, *122*, *131*, *137*, *143*, *149*, *154*, *160*, *166*, *173* and *178*). In applying the Procedure, the chemical is first assigned to a structural class as identified by the Committee at its forty-sixth meeting (Annex 1, reference *122*). The structural classes are as follows:

- *Class I.* Flavouring agents that have simple chemical structures and efficient modes of metabolism that would suggest a low order of toxicity by the oral route.
- *Class II.* Flavouring agents that have structural features that are less innocuous than those of substances in class I but are not suggestive of toxicity. Substances in this class may contain reactive functional groups.
- *Class III.* Flavouring agents that have structural features that permit no strong initial presumption of safety or may even suggest significant toxicity.

A key element of the Procedure involves determining whether a flavouring agent and the product(s) of its metabolism are innocuous and/or endogenous substances. For the purpose of the evaluations, the Committee used the following definitions, adapted from the report of its forty-sixth meeting:

- *Innocuous metabolic products a*re defined as products that are known or readily predicted to be harmless to humans at the estimated intake of the flavouring agent.
- *Endogenous substances a*re intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included. The estimated intake of a flavouring agent that is, or is metabolized to, an endogenous substance should be judged not to give rise to perturbations outside the physiological range.

Estimates of the intake of flavouring agents by populations typically involve the acquisition of data on the amounts used in food. These data were derived from surveys in Europe, Japan and the USA. In Europe, a survey was conducted in 1995 by the International Organization of the Flavor Industry, in which flavour manufacturers reported the total amount of each flavouring agent incorporated into food sold in the EU during the previous year.

Manufacturers were requested to exclude use of flavouring agents in pharmaceutical, tobacco or cosmetic products.

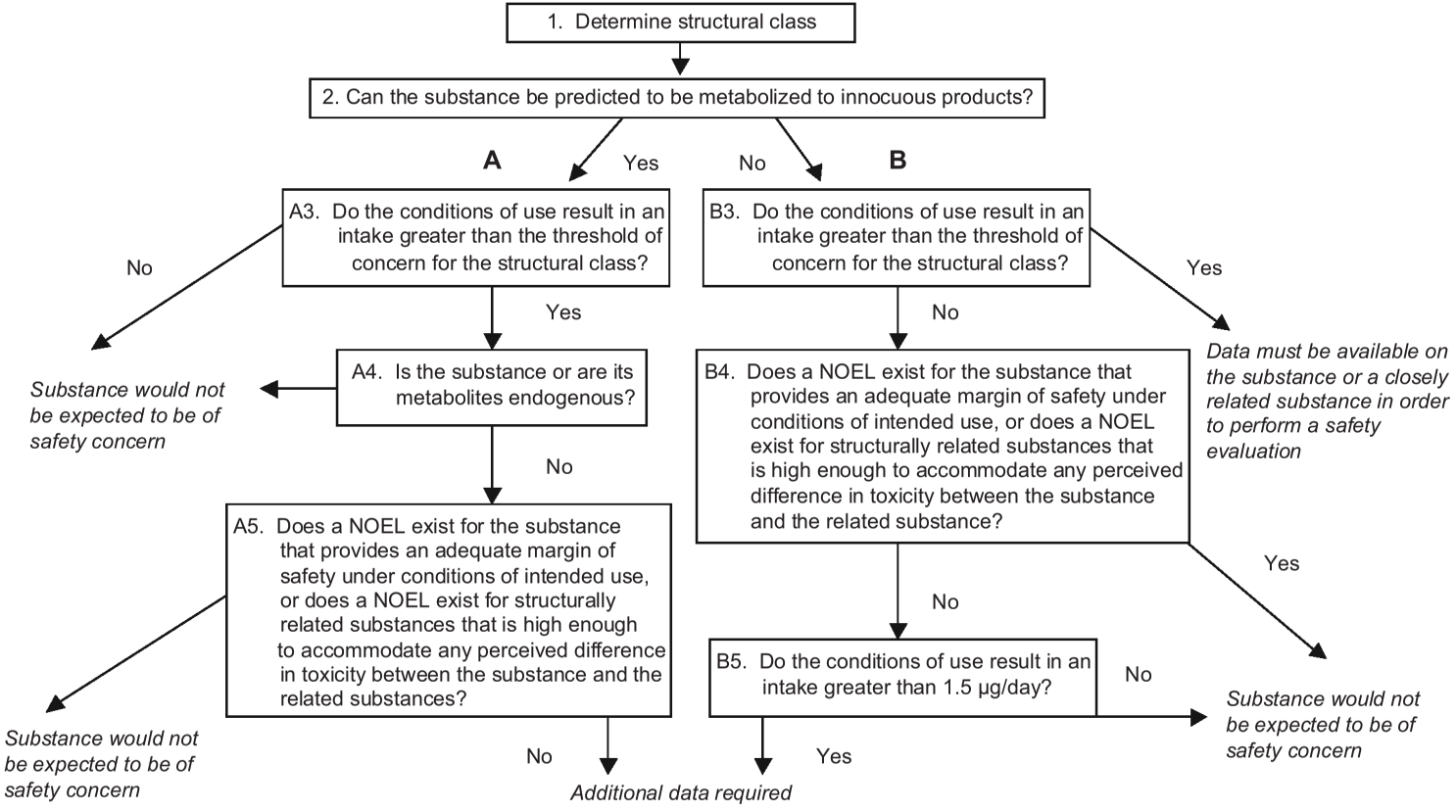
In the USA, a series of surveys was conducted between 1970 and 1987 by the National Research Council of the National Academy of Sciences (under contract to the Food and Drug Administration) in which information was obtained from ingredient manufacturers and food processors on the amount of each substance destined for addition to the food supply and on the usual and maximal levels at which each substance was added in a number of broad food categories.

In using the data from these surveys to estimate intakes of flavouring agents, it was previously assumed that only 60% of the total amount used is reported in the USA and 80% of the amount used is reported in Europe and that the total amount used in food is consumed by only 10% of the population. At the present meeting, a correction factor of 0.8 was applied to the annual production volumes reported in the recent surveys from Europe, Japan and the USA.

$$\text{Intake (µg/person per day)} = \frac{\text{annual volume of production (kg)} \times 10^9 \text{ (µg/kg)}}{\text{population of consumers} \times 0.6 \text{ (or } 0.8) \times 365 \text{ days}}$$

The population of consumers was assumed to be 32×10^6 in Europe, 13×10^6 in Japan and 28×10^6 in the USA.

Figure 1 Procedure for the Safety Evaluation of Flavouring Agents



ALIPHATIC ACYCLIC AND ALICYCLIC TERPENOID TERTIARY ALCOHOLS AND STRUCTURALLY RELATED SUBSTANCES (addendum)

First draft prepared by Professor I.G. Sipes[1] and Professor J. Bend[2]

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 15 aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances that included 3 terpene alcohols, 3 aliphatic tertiary alcohols, 3 phenyl-substituted aliphatic alcohols, 4 esters of phenyl-substituted aliphatic alcohols and 2 spiranes. The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, Introduction) (Annex 1, reference *131*). None of these agents has previously been evaluated.

The Committee previously evaluated 23 other members of this chemical group of flavouring agents at its fifty-first meeting (Annex 1, reference *137*). The findings presented in that report were considered in the present evaluation. Twenty-two of the 23 substances evaluated at the fifty-first meeting were concluded to be

of no safety concern based on currently estimated levels of intake. The Committee concluded that additional data were required for the evaluation of methyl 1-acetoxycyclohexylketone (No. 442).

Five of the 15 flavouring agents evaluated in the current group are natural components of foods (Nos 1646 and 1650–1653). They have been detected in a wide variety of foods, including pepper, orange juice and peel, lemon juice, grapefruit juice, berries, pineapple, guava, melon, tomato, mango, beer, sage, parsley, lemon balm, beans, rice, ginger, cocoa, black and green teas, red and white wines, brandy, mango, peppermint oil, spearmint oil, skim milk powder, a variety of other herbs and spices and a number of citrus oils and honeys (Nijssen et al., 2006).

1.2 Assessment of dietary exposure

The total annual volume of production of this group of aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances is approximately 1800 kg in Europe, 13 000 kg in the United States of America (USA) and 960 kg in Japan (National Academy of Sciences, 1970; Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006). Greater than 90% of the annual production volume in Europe, the USA and Japan is accounted for by α,α-dimethylphenethyl acetate (No. 1655) and α,α-dimethylphenethyl butyrate (No. 1656). The daily per capita intake of each agent is reported in Table 1. Annual volumes of production of this group of flavouring agents are summarized in Table 2.

1.3 Absorption, distribution, metabolism and elimination

It is anticipated that the esters in this group would be readily hydrolysed to their component alcohols and carboxylic acids (Heymann, 1980; Anders, 1989). The hydrolysis products would be readily metabolized primarily by conjugation with glucuronic acid and are excreted primarily in the urine (Williams, 1959; Parke et al., 1974; Horning et al., 1976; Ventura et al., 1985). Alternatively, alcohols with unsaturation may be ω-oxidized at the allylic position to yield polar metabolites, which may be conjugated and excreted. Metabolites of acyclic alcohols may be further oxidized to eventually yield carbon dioxide.

Table 1. Summary of the results of safety evaluations of aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances used as flavouring agents[a,b,c]

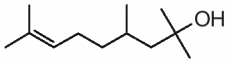
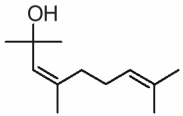
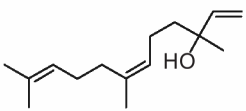
Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
Structural class I					
2,3,4-Trimethyl-3-pentanol	1643	3054-92-0 	No Europe: 0.04 USA: ND Japan: ND	See note 1	No safety concern
(±)-2,4,8-Trimethyl-7-nonen-2-ol	1644	437770-28-0 	No Europe: 0.01 USA: 0.1 Japan: ND	See note 2	No safety concern
trans- and *cis*-2,4,8-Trimethyl-3,7-nonadien-2-ol	1645	479547-57-4 	No Europe: 0.01 USA: 0.1 Japan: ND	See note 2	No safety concern
Nerolidol	1646	7212-44-4 	No Europe: 38 USA: 23 Japan: 87	See note 2	No safety concern

Table 1 (contd)

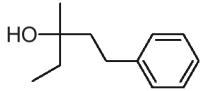
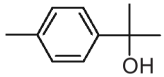
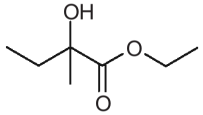
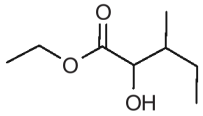
Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
1-Phenyl-3-methyl-3-pentanol	1649	10415-87-9 	No Europe: 0.01 USA: 0.1 Japan: 0.9	See note 1	No safety concern
p-α,α-Trimethylbenzyl alcohol	1650	1197-01-9 	No Europe: 9 USA: 0.01 Japan: 0.7	See note 1	No safety concern
(±)-Ethyl 2-hydroxy-2-methylbutyrate	1651	77-70-3 	No Europe: ND USA: 0.6 Japan: ND	See note 1	No safety concern
(±)-Ethyl 2-hydroxy-3-methylvalerate	1652	24323-38-4 	No Europe: ND USA: 0.2 Japan: ND	See note 1	No safety concern

Table 1 (contd)

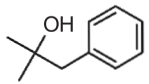
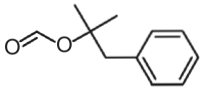
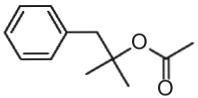
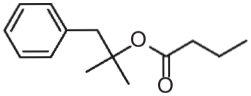
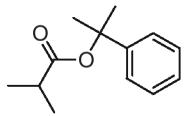
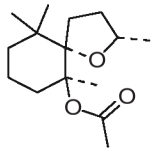
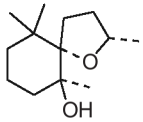
Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
α,α-Dimethylphenethyl alcohol	1653	100-86-7 	No Europe: 17 USA: 12 Japan: 16	See note 1	No safety concern
α,α-Dimethylphenethyl formate	1654	10058-43-2 	No Europe: ND USA: 0.4 Japan: 0.2	See note 3	No safety concern
α,α-Dimethylphenethyl acetate	1655	151-05-3 	No Europe: 41 USA: 574 Japan: 50	See note 3	No safety concern
α,α-Dimethylphenethyl butyrate	1656	10094-34-5 	No Europe: 93 USA: 1020 Japan: 98	See note 3	No safety concern

Table 1 (contd)

Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
α,α-Dimethylbenzyl isobutyrate	1657	7774-60-9 	No Europe: 0.01 USA: ND Japan: 0.03	See note 3	No safety concern
Structural class II					
6-Acetoxydihydrotheaspirane	1647	57893-27-3 	No Europe: 0.07 USA: ND Japan: 0.03	See note 3	No safety concern
6-Hydroxydihydrotheaspirane	1648	65620-50-0 	No Europe: 0.1 USA: 0.05 Japan: 0.03	See note 3	No safety concern

CAS, Chemical Abstracts Service; ND, no data reported.

[a] Twenty-three flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *137*).

[b] *Step 1*: Thirteen flavouring agents are in structural class I, and two (Nos 1647 and 1648) are in structural class II.

[c] *Step 2*: All of the flavouring agents in this group can be predicted to be metabolized to innocuous products.
[d] The thresholds for human intake for structural classes I and II are 1800 and 540 µg/day, respectively. All intake values are expressed in µg/day.

Notes:
1. Tertiary alcohols are metabolized primarily by conjugation with glucuronic acid and excreted in the urine.
2. Tertiary unsaturated alcohols are metabolized primarily by conjugation with glucuronic acid and excreted in the urine. Oxidation of the allylic methyl group may occur at high doses.
3. Esters are hydrolysed, and the corresponding tertiary alcohols are metabolized primarily by conjugation with glucuronic acid and excreted in the urine.

Table 2. Annual volumes of production of aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
2,3,4-Trimethyl-3-pentanol (1643)					
Europe	0.4	0.04	0.0007		
USA	ND	ND	ND	–	NA
Japan	ND	ND	ND		
(±)-2,4,8-Trimethyl-7-nonen-2-ol (1644)					
Europe	0.1	0.01	0.0002		
USA	1	0.1	0.002	–	NA
Japan	ND	ND	ND		
trans- and *cis*-2,4,8-Trimethyl-3,7-nonadien-2-ol (1645)					
Europe	0.1	0.01	0.0002		
USA	1	0.1	0.002	–	NA
Japan	ND	ND	ND		
Nerolidol (1646)					
Europe	354	38	0.6		
USA	186	23	0.4	300 000	1613
Japan	331	87	1		

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
6-Acetoxydihydrotheaspirane (1647)					
Europe	0.7	0.07	0.001		
USA	ND	ND	ND	–	NA
Japan	0.1	0.03	0.0004		
6-Hydroxydihydrotheaspirane (1648)					
Europe	1	0.1	0.002		
USA	0.4	0.05	0.0008	–	NA
Japan	0.1	0.03	0.0004		
1-Phenyl-3-methyl-3-pentanol (1649)					
Europe	0.1	0.01	0.0002		
USA	1	0.1	0.002	–	NA
Japan	3	0.9	0.02		
p-α,α-Trimethylbenzyl alcohol (1650)					
Europe	79	9	0.2		
USA	0.1	0.01	0.0002	500 000	5 000 000
Japan	3	0.7	0.01		

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
(±)-Ethyl 2-hydroxy-2-methylbutyrate (1651)					
Europe	ND	ND	ND		
USA	5	0.6	0.01	+	NA
Japan	ND	ND	ND		
(±)-Ethyl 2-hydroxy-3-methylvalerate (1652)					
Europe	ND	ND	ND		
USA	2	0.2	0.004	+	NA
Japan	ND	ND	ND		
α,α-Dimethylphenethyl alcohol (1653)					
Europe	160	17	0.3		
USA	98	12	0.2	+	NA
Japan	59	16	0.3		
α,α-Dimethylphenethyl formate (1654)					
Europe	ND	ND	ND		
USA[e]	3	0.4	0.007	–	NA
Japan	0.6	0.2	0.003		

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
α,α-Dimethylphenethyl acetate (1655)					
Europe	384	41	0.7		
USA	4696	574	10	–	NA
Japan	191	50	0.8		
α,α-Dimethylphenethyl butyrate (1656)					
Europe	869	93	2		
USA	8343	1020	17	–	NA
Japan	371	98	2		
α,α-Dimethylbenzyl isobutyrate (1657)					
Europe	0.1	0.01	0.0002		
USA	ND	ND	ND	–	NA
Japan	0.1	0.03	0.0004		
Total					
Europe	1849				
USA	13 336				
Japan	959				

bw, body weight; NA, not available; ND, no intake data reported; +, reported to occur naturally in foods (Nijssen et al., 2006), but no quantitative data; −, not reported to occur naturally in foods.

[a] From European Flavour and Fragrance Association (2005), Flavor and Extract Manufacturers Association (2006) and Japanese Flavor and Fragrance Manufacturers Association (2002). Total poundage values of <1 kg reported in the surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006) have been truncated to one place following the decimal point (0.1 kg).

[b] Intake (μg/person per day) calculated as follows:
[(annual volume, kg) × (1×10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = 32×10^6 for Europe, 28×10^6 for the USA and 13×10^6 for Japan; and where correction factor = 0.6 for National Academy of Sciences survey in the USA and 0.8 for the surveys by the USA, Europe and Japan, representing the assumption that only 60% and 80% of the annual flavour volume, respectively, were reported in the poundage surveys (NAS, 1970; Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006).
Intake (μg/kg bw per day) calculated as follows:
(μg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur as a result of rounding.

[c] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[d] The consumption ratio is calculated as follows:
(annual consumption via food, kg)/(most recent reported volume as a flavouring substance, kg)

[e] Annual volume reported in previous USA surveys (NAS, 1970).

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned all but two flavouring agents to structural class I (Cramer et al., 1978). 6-Acetoxydihydrotheaspirane (No. 1647) and 6-hydroxydihydrotheaspirane (No. 1648) were assigned to structural class II (Cramer et al., 1978).

Step 2. All flavouring agents in this group are expected to be metabolized to innocuous products. The evaluation of all the substances in this group therefore proceeded via the A-side of the Procedure.

Step A3. The estimated daily per capita intakes for the 13 flavouring agents in structural class I are below the threshold of concern (i.e. 1800 µg/person per day for class I). The estimated daily per capita intakes for the 2 flavouring agents in structural class II are below the threshold of concern (540 µg/person per day for class II). According to the Procedure, the safety of these 15 flavouring agents raises no concern when they are used at their current estimated levels of intake.

Table 1 summarizes the evaluations of the 15 aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances in this group.

1.5 Consideration of combined intakes from use as flavouring agents

The flavouring agents in this group are predicted to be metabolized by hydrolysis and/or oxidative metabolism of alcohol and aldehyde groups and alkyl side-chains, by conjugation reactions such as glucuronidation and possibly by aromatic hydroxylation. These pathways have a high capacity and would not be saturated, even if all agents were consumed at the same time. Some of the substances in this group that have been evaluated at this meeting and at the fifty-first and sixty-first meetings are predicted to be metabolized to common metabolites. Common metabolites (and their precursors) are linalool (Nos 356 and 358–365), terpineol (Nos 366–372), α,α-dimethylphenyl alcohol (Nos 1653–1657) and 6-hydroxydihydrotheaspirane (Nos 1647 and 1648). The estimated combined intake of substances predicted to be metabolized to α,α-dimethylphenyl alcohol was below the threshold for class I, and the combined intake of acetoxydihydrotheaspirane and 6-hydroxydihydrotheaspirane (Nos 1647 and 1648) was below the threshold for class II. In the case of linalool, the total combined intake did not add significantly to the combined intake of linalool (No. 356) and linalyl acetate (No. 359), which exceeded the class I threshold. This combined intake was evaluated at the fifty-first meeting and was considered not to be a safety concern. In the case of terpineol, the total combined intake did not add significantly to the combined intake of terpineol (No. 366) and terpinyl acetate (No. 368), which exceeded the class I threshold. This combined intake was evaluated at the fifty-first meeting and was considered not to be a safety concern. The Committee concluded that under the conditions of use as

flavouring agents, the combined intake of the substances leading to a common metabolite would not saturate the metabolic pathways and the combined intakes would not raise safety concerns.

1.6 Consideration of secondary components

Two members of this group of flavouring agents, *p*-α,α-trimethylbenzyl alcohol (No. 1650) and α,α-dimethylphenethyl formate (No. 1654), have assay values of <95%. Information on the safety of the secondary components of these two compounds is summarized in Annex 5 (Summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%). The secondary component of *p*-α,α-trimethylbenzyl alcohol, *p*-α-dimethylstyrene (No. 1333), is expected to undergo cytochrome P450–mediated metabolism followed by hydrolysis/conjugation. It is considered not to present a safety concern at current levels of intake. The secondary component of α,α-dimethylphenethyl formate, α,α-dimethylphenethyl alcohol, is expected to share the same metabolic fate as the ester and is considered not to present a safety concern at current levels of intake.

1.7 Conclusion

In the previous evaluations of substances in this group, studies of acute toxicity, short-term toxicity (84–149 days), carcinogenicity and genotoxicity were available. None raised safety concerns. The data available for this evaluation were supported by those from the previous evaluation.

The Committee concluded that these 15 flavouring agents, which are additions to the group of aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances evaluated previously, would not give rise to safety concerns at the currently estimated levels of intake.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of 15 aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances (see Table 1), which are additions to the group of flavouring agents evaluated previously.

2.2 Additional considerations on intake

There is no additional information on intake.

2.3 Biological data

2.3.1 Biochemical data: hydrolysis, absorption, distribution, metabolism and excretion

As described in the fifty-first meeting report (Annex 1, reference *137*), aliphatic acyclic and alicyclic terpenoids and related esters undergo efficient metabolism. Terpene esters including esters formed from tertiary alcohols (i.e. linalool) and simple aliphatic carboxylic acids undergo hydrolysis. Results of studies in aqueous buffered conditions, simulated gastric juice, simulated human intestinal fluid, blood plasma, whole hepatocytes and liver microsome preparations demonstrate this. Although differences in the rates of hydrolysis occur under in vitro conditions in gastric juice and intestinal fluids, ready hydrolysis is observed in tissue preparations rich in carboxyl esterase, including blood and liver.

Esters that survive intestinal fluids and blood intact are subject to rapid hydrolysis in the liver. For example, α,α-dimethylphenethyl acetate (No. 1655) is resistant to hydrolysis in most in vitro conditions, but undergoes hydrolysis in liver preparations (Leegwater & van Straten, 1974; Grundschober, 1977).

Based on these data, it is concluded that monoterpene esters will undergo in vivo hydrolysis in animals to yield the corresponding alcohols and carboxylic acids. Given that the carboxylesterases and lipases catalysing ester hydrolysis are present in all animals, including fish, it is concluded that monoterpene esters will be hydrolysed to yield monoterpene alcohols and simple aliphatic and aromatic acids. Once hydrolysed, the component alcohols and acids are subject to further oxidative metabolism and/or conjugation.

It is expected that the tertiary aromatic alcohols in this group of flavouring agents and those formed via hydrolysis will undergo direct conjugation of the hydroxyl group with glucuronic acid (Williams, 1959). In rabbits administered dimethylphenylcarbinol (No. 203), a structurally similar compound, 85% of the original dose was excreted in the urine as the corresponding glucuronic acid conjugate (Robinson et al., 1955).

Terpenoids may affect the activity of other hepatic enzymes. Such effects would be dependent on dose, route of administration and the species under study. Minor effects were observed when rats were administered a series of terpenoids, including nerolidol (No. 1646), by intraperitoneal injection or as an admixture to the feed for 3 days. Biphenyl 4-hydroxylation, glucuronyl transferase and 4-nitrobenzoate reduction activities and cytochrome P450 levels were determined in liver homogenates following treatment. Nerolidol produced no increase in enzymatic activities for biphenyl 4-hydroxylation, glucuronyl transferase or cytochrome P450s, but increased 4-nitrobenzoate reduction activity by 25–50% above background levels (Parke & Rahman, 1969).

2.3.2 Toxicological studies

(a) Acute toxicity

Oral LD_{50} values have been reported for several substances in this group and are summarized in Table 3. In rats, LD_{50} values range from 1280 mg/kg bw for α,α-dimethylphenethyl alcohol (No. 1653) to 6960 mg/kg bw for 6-acetoxydihydrotheaspirane (No. 1647), demonstrating that the oral acute toxicity of these tertiary alcohols is low (Jenner et al., 1964; Moreno, 1973, 1975, 1977; Russell, 1973; Griffiths, 1979; Collinson, 1989; Moore, 2000).

LD_{50} values reported in mice (9976 mg/kg bw) and guinea-pigs (988 mg/kg bw) for nerolidol (No. 1646) and α,α-dimethylphenethyl alcohol (No. 1653), respectively, confirm the low acute toxicity of these agents (Jenner et al., 1964; Colaianni, 1967).

(b) Short-term studies of toxicity

Short-term studies of toxicity have been performed on four flavouring agents in the present group. The results of these tests are summarized in Table 4 and are described below.

Table 3. Results of oral acute toxicity studies with aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1646	Nerolidol	Rat; NR	>5000	Russell (1973)
1646	Nerolidol	Mouse; M	9976	Colaianni (1967)
1647	6-Acetoxydihydro theaspirane	Rat; MF	6960	Griffiths (1979)
1649	1-Phenyl-3-methyl-3-pentanol	Rat; NR	2950	Moreno (1975)
1653	α,α-Dimethylphenethyl alcohol	Guinea-pig; MF	988	Jenner et al. (1964)
1653	α,α-Dimethylphenethyl alcohol	Rat; NR	1350	Moreno (1973)
1653	α,α-Dimethylphenethyl alcohol	Rat; MF	1280	Jenner et al. (1964)
1654	α,α-Dimethylphenethyl formate	Rat; MF	>2000	Collinson (1989)
1655	α,α-Dimethylphenethyl acetate	Rat; M	3300	Moreno (1971)
1656	α,α-Dimethylphenethyl butyrate	Rat; NR	>5000	Moreno (1977)
1650	*p*-α,α-Trimethylbenzyl alcohol	Rat; NR	>2000	Moore (2000)

F, female; M, male; NR, not reported.

Table 4. Results of short-term studies of toxicity with aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a] / no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1643	2,3,4-Trimethyl-3-pentanol	Rat; MF	1/10	Diet	14	10[c]	Madarasz (1997)
1647	6-Acetoxydihydro-theaspirane	Rat; MF	1/32	Gavage	91	3[c,d]	Griffiths (1979)
1648	6-Hydroxydihydro-theaspirane	Rat; MF	1/32	Gavage	91	0.154[c]	Griffiths (1976)
1653	α,α-Dimethyl-phenethyl alcohol	Rat; MF	1/10	Diet	112	1000[c,e]	Hagan et al. (1967)
1653	α,α-Dimethyl-phenethyl alcohol	Rat; MF	1/20	Diet	196	100[c,f]	Hagan et al. (1967)

F, female; M, male; NOEL, no-observed-effect level.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] Study performed with either a single dose or multiple doses that had no adverse effect; the value is therefore not a true NOEL, but is the highest dose level tested that produced no adverse effects. The actual NOEL may be higher.

[d] Compared with controls, a significant increase was observed in relative spleen weights in males; however, this organ weight variation was not accompanied by any histopathological abnormalities or any other adverse effects.

[e] Based on a dietary concentration of 10 000 mg/kg, which is equivalent to 1000 mg/kg bw.

[f] Based on a dietary concentration of 1000 mg/kg, which is equivalent to 100 mg/kg bw.

(i) 2,3,4-Trimethyl-3-pentanol (No. 1643)

Groups of Sprague-Dawley rats (five per sex) were maintained on diets providing 2,3,4-trimethyl-3-pentanol at a dose level of 0 or 10 mg/kg bw per day for 14 days. Animals were observed daily for signs of gross toxicity. Body weight was measured on days 7 and 14 of the study. Food consumption was calculated based on a 6-day interval between days 1 and 7 and between days 8 and 14. On day 15, gross necropsies were performed on all animals, and kidneys and livers of each animal were removed, weighed and prepared for histological evaluation. All animals in the study survived and appeared healthy. Body weight gain, food consumption and organ to body weight ratios were not significantly different between test and control animals. Gross necropsy and histopathological examination of kidney and liver tissues revealed no lesions related to administration of the test material. Test

and control groups showed no significant differences in relative or absolute kidney or liver weights (Madarasz, 1997).

(ii) 6-Acetoxydihydrotheaspirane (No. 1647)

In a study using groups of 16 Sprague-Dawley rats per sex per dose, 6-acetoxydihydrotheaspirane was administered at a dose of 3 mg/kg bw per day for 91 days. Animals were housed four per cage, and food and water were available ad libitum. Body weight and food consumption were monitored weekly, and animals were observed daily for ill health or behavioural changes. At 4 and 13 weeks, blood was drawn from eight male and eight female animals for haematological evaluation. At the conclusion of the study, all animals were sacrificed, and a complete necropsy was performed. Tissue samples of major organs were taken for histological examination. Treated females were reported to have significantly decreased body weights compared with controls at week 13. In comparison with controls, a significant increase in relative spleen weight was observed in treated males. Significant reductions in haemoglobin, haematocrit and red blood cell count were observed in treated females relative to levels observed in controls. No other effects were reported (Griffiths, 1979).

(iii) 6-Hydroxydihydrotheaspirane (No. 1648)

Groups of 16 male and 16 female weanling Wistar rats were administered 6-hydroxydihydrotheaspirane at 0.154 mg/kg bw per day in corn oil, by gavage, for 13 weeks. Control animals were given only corn oil. Animals were housed two of the same sex per cage in a room maintained at 20 ± 2 ºC at a relative humidity of 40–60% and a daily cycle of 12 h light/12 h dark. Food and water were available ad libitum. The animals were weighed 5 days and 1 day before treatment, on the first day of dosing (day 0) and thereafter at regular intervals throughout the experiment. The food intake was recorded at 3- or 4-day intervals throughout the experiment. Blood was collected from the tail vein of half the rats (one from each cage of two) at week 6 and all animals at week 12. Blood was examined for haemoglobin concentration, packed cell volume and counts of erythrocytes and leukocytes (total and differential). In addition, urea concentration was measured. At the end of the exposure period, animals were fasted overnight, killed and subjected to a postmortem examination, during which any abnormalities were noted and the liver and kidneys were weighed. Samples of major organs were also preserved in formalin for histopathological examination. No treatment-related clinical effects were observed. One male rat died during the study. The death occurred within 1 h of dosing, and necropsy revealed oil in the lungs and trachea, but not in the upper part of the gastrointestinal tract. At week 12, treated males had significantly greater haemoglobin concentrations relative to controls. At week 6, but not at week 12, treated males and females had significantly greater total leukocyte counts compared with the controls (Griffiths, 1976).

(iv) α,α-Dimethylphenethyl alcohol (No. 1653)

Groups of five weanling Osborne-Mendel rats per sex were housed individually in wire cages and administered 0 or 10 000 mg α,α-dimethylphenethyl alcohol/kg in the diet (approximately 1000 mg/kg bw per day) for 16 weeks. In addition, groups of 10 rats per sex were administered 0 or 1000 mg α,α-dimethylphenethyl alcohol/kg in the diet (approximately 100 mg/kg bw per day) for 28 weeks. Both sets of rats were allowed food and water ad libitum, and the diet was prepared weekly. Body weight, food intake and general condition were recorded weekly. Haematological examinations, which included white cell counts, red cell counts and levels of haemoglobin and haematocrit, were conducted at the termination of the study. On completion of the study, all surviving animals were necropsied and examined macroscopically. Organ weights were recorded, and tissues were preserved for histopathological examination. Detailed microscopic examinations were done on six or eight animals evenly divided by sex, from the treatment group and the control group. No effects were observed due to the administration of the test material at either dose level (Hagan et al., 1967).

(c) Genotoxicity

Genotoxicity testing has been performed on two substances in the present group. The results of these tests are summarized in Table 5 and are described below.

(i) In vitro

Negative results were reported in the Ames assay for 1-phenyl-3-methyl-3-pentanol (No. 1649) and α,α-dimethylphenethyl formate (No. 1654) tested at concentrations of up to 3.6 and 1.0 mg/plate, respectively, with and without metabolic activation in several strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA1538) (Wild et al., 1983; Asquith, 1989).

The absence of mutagenic activity related to terpenoids is further supported by the negative results reported for two structurally similar flavouring agents, linalyl acetate (No. 359) and linalyl propionate (No. 360), which have been previously evaluated by the Committee. No mutagenic potential was observed when *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were incubated with 0, 33, 100, 333, 1000 or 5000 µg linalyl propionate/plate in the presence or absence of metabolic activation (Sokolowski, 2004). No chromosomal aberrations were observed when linalyl acetate at 0, 10, 33, 56, 100, 130 or 180 µg/ml was incubated with human peripheral lymphocytes for 3 h with 24- and 48-h fixation times, with or without metabolic activation (Bertens, 2000).

(ii) In vivo

The potential of 1-phenyl-3-methyl-3-pentanol (No. 1649) to induce sex-linked recessive lethal mutations in adult *Drosophila melanogaster* was studied in the Basc test. Mutation frequency was unaffected when flies were exposed to

Table 5. Studies of genotoxicity with aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
1649	1-Phenyl-3-methyl-3-pentanol	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	Up to 3600 µg/plate	Negative[a]	Wild et al. (1983)
1654	α,α-Dimethyl-phenethyl formate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	Up to 1000 µg/plate	Negative[a]	Asquith (1989)
In vivo						
1649	1-Phenyl-3-methyl-3-pentanol	Sex-linked recessive lethal mutation (Basc test)	*Drosophila melanogaster*	0 or 20 mmol/l (3565 µg/ml)[b]	Negative	Wild et al. (1983)
1649	1-Phenyl-3-methyl-3-pentanol	Micronucleus induction	NMRI mice	0, 357, 624, 891 or 1416 mg/kg bw	Negative	Wild et al. (1983)

[a] With and without metabolic activation.
[b] Calculated using molecular weight of 1-phenyl-3-methyl-3-pentanol = 178.28.

a 0 or 20 mmol/l (3565 µg/ml) solution of 1-phenyl-3-methyl-3-pentanol for 3 days (Wild et al., 1983).

In a micronucleus test, groups of four male and female NMRI mice (number per sex not reported), administered single intraperitoneal doses of 0, 357, 624, 891 or 1416 mg 1-phenyl-3-methyl-3-pentanol (No. 1649)/kg bw, demonstrated no increase in micronucleated erythrocytes in bone marrow samples obtained 30 h post-administration (Wild et al., 1983).

(iii) Conclusion for genotoxicity

The testing of these representative materials in vitro in bacterial test systems (Ames assay) and in vivo in mammalian (micronucleus test) systems showed no evidence of mutagenic or genotoxic potential. These results are further supported by the lack of positive findings in the Basc test.

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SIMPLE ALIPHATIC AND AROMATIC SULFIDES AND THIOLS (addendum)

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of flavouring substances consisting of 51 simple aliphatic and aromatic sulfides and thiols, which included 3 simple sulfides (Nos 1683, 1684 and 1707), 10 acyclic sulfides with oxidized and thiol side-chains (Nos 1668, 1675, 1677, 1688–1692, 1703 and 1710), 1 heterocyclic sulfide (No. 1685), 5 thiols (Nos 1659 and 1662–1665), 12 thiols with oxidized side-chains (Nos 1666, 1667, 1669–1674, 1704–1706 and 1708), 3 dithiols (Nos 1660, 1661 and 1709), 7 disulfides (Nos 1693, 1694 and 1696–1700), 2 trisulfides (Nos 1695 and 1701), 2 heterocyclic disulfides (Nos 1686 and 1687) and 6 thioesters and acids (Nos 1676, 1678–1681 and 1702). The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, Introduction) (Annex 1, reference *131*). None of these substances has previously been evaluated.

The Committee previously evaluated 137 other members of this chemical group of flavouring agents at its fifty-third meeting (Annex 1, reference *143*). The findings from these evaluations were considered in the present evaluation. All 137 substances in that group were concluded to be of no safety concern based on currently estimated levels of intake.

The Committee also evaluated 12 additional members of this chemical group of flavouring agents at its sixty-first meeting (Annex 1, reference *166*). The findings from these evaluations were considered in the present evaluation. All 12 additional substances in that group were concluded to be of no safety concern based on currently estimated levels of intake.

Thirty-seven of the 51 flavouring agents in this group are natural components of foods (Nos 1659, 1660, 1662–1665, 1667, 1668, 1674, 1676–1678, 1680, 1683–1687, 1690–1704, 1706, 1707, 1709 and 1710). They have been detected primarily in beef, chicken, pork, fish, lobster, cheese, eggs, grapefruit juice, coffee, cabbage, onion, scallions, garlic, potato, tomato, melon, papaya, pineapple, kiwifruit, chive, nobiru, kohlrabi, wakegi, leek, caucas, strawberry, hop oil, beer, wine, rum, filbert, hazelnut, arrack, fish oil, carrot, sauerkraut, trassi, sweet corn, sesame seed, passion fruit and durian fruit (Boelens et al., 1971; Freeman & Whenham, 1976; Ishikawa et al., 1978; Demole et al., 1982; Tada et al., 1988; Kuo & Ho, 1992; Kumazawa et al., 1998; Tominaga & Dubourdieu, 2000; Kodera et al., 2002; Kumazawa & Masuda, 2003; Flavor and Extract Manufacturers Association, 2006; Nijssen et al., 2006). Quantitative intake data were available for two substances, 1-pentanethiol (No. 1662) and methyl (methylthio)acetate (No. 1691). The

consumption ratios (the ratios of their consumption from natural food sources to their use as flavouring agents) were calculated to be 106 and 37, respectively.

1.2 Assessment of dietary exposure

The total annual volume of production of the 51 simple aliphatic and aromatic sulfides and thiols is approximately 182 kg in Europe (European Flavour and Fragrance Association, 2005), 80 kg in the United States of America (USA) (Flavor and Extract Manufacturers Association, 2006) and 3 kg in Japan (Japanese Flavor and Fragrance Manufacturers Association, 2002). In Europe, approximately 72% of the total volume is accounted for solely by diethyl trisulfide (No. 1701), whereas in the USA, (*S*)-1-methoxy-3-heptanethiol (No. 1671), (±)-isobutyl 3-methylthiobutyrate (No. 1677), methyl (methylthio)acetate (No. 1691), bis(1-mercaptopropyl)sulfide (No. 1709) and *S*-allyl-L-cysteine (No. 1710) account for 84% of the total volume of production. The daily per capita intake of each agent is reported in Table 1. Annual volumes of production of this group of flavouring agents are summarized in Table 2.

1.3 Absorption, distribution, metabolism and elimination

All of the sulfur-containing flavouring agents reviewed here are of low molecular weight and are sufficiently lipophilic to be absorbed. These flavouring agents are expected to be metabolized through the various pathways described below and in the previous evaluations by the Committee (Annex 1, references *143* and *166*).

1.3.1 Simple sulfides (Nos 1683, 1684 and 1707)

Once simple sulfides (thioethers) enter the systemic circulation, they are rapidly oxidized to sulfoxides and, depending on the structure of the thioether, may be further oxidized to sulfones. Aliphatic thioethers yield mixtures of sulfoxide and sulfone urinary metabolites (Damani, 1987). Enzymes of the cytochrome P450 superfamily and flavin-containing monooxygenases catalyse the oxidation of thioethers to sulfoxides (Renwick, 1989). Oxidation of sulfoxides to the corresponding sulfones occurs both in tissues and in aerobic microorganisms and is an irreversible metabolic reaction in mammals (Damani, 1987). Sulfoxides can also be metabolized back to the thioether by thioredoxin and its reductase and by the gut microflora in the anaerobic environment of the lower bowel (Lee & Renwick, 1995).

1.3.2 Acyclic sulfides with oxidized or thiol side-chains (Nos 1668, 1675, 1677, 1688–1692, 1703 and 1710)

Hemiacetal derivatives of thiols (No. 1675) would be expected to undergo metabolism via the pathways described above for simple thiols. The thioether sulfur and the free thiol can undergo oxidation as described in more detail below, and methylation of the free thiol could also occur. The presence of oxygenated functional groups, such as an alcohol (No. 1703), aldehyde (No. 1692), acid (No. 1710),

Table 1. Summary of the results of safety evaluations of simple aliphatic and aromatic sulfides and thiols used as flavouring agents [a,b,c]

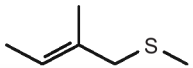
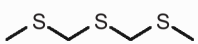
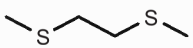
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Simple sulfides						
Structural class I						
2-Methyl-1-methylthio-2-butene	1683	89534-74-7 	No Europe: 0.01 USA: 0.1 Japan: ND	Yes. The NOEL of 250 mg/kg bw per day (Butterworth et al., 1975) for the related substance methyl sulfide (No. 452) is at least 125 million times the estimated daily intake of 2-methyl-1-methylthio-2-butene when used as a flavouring agent.	See note 1	No safety concern
2,4,6-Trithiaheptane	1684	6540-86-9 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 250 mg/kg bw per day (Butterworth et al., 1975) for the related substance methyl sulfide (No. 452) is >1 billion times the estimated daily intake of 2,4,6-trithiaheptane when used as a flavouring agent.	See note 1	No safety concern
2,5-Dithiahexane	1707	6628-18-8 	No Europe: ND USA: 0.1 Japan: ND	Yes. The NOEL of 250 mg/kg bw per day (Butterworth et al., 1975) for the related substance methyl sulfide (No. 452) is 125 million times the estimated daily intake of 2,5-dithiahexane when used as a flavouring agent.	See note 1	No safety concern

Table 1 (contd)

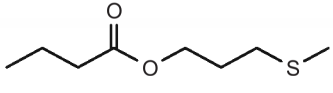
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Acyclic sulfides with oxidized and thiol side-chains						
Structural class I						
Methionyl butyrate	1668	16630-60-7 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 1.4 mg/kg bw per day for the related substance 2-(methylthiomethyl)-3-phenylpropenal (No. 505) (Cox et al., 1978) is 7 million times the estimated daily intake of methionyl butyrate when used as a flavouring agent.	See notes 1, 2 and 3	No safety concern
Methylthiomethyl-mercaptan	1675	29414-47-9 	No Europe: 0.01 USA: 0.1 Japan: ND	Yes. The NOEL of 0.3 mg/kg bw per day for the related substance 3-methyl-1,2,4-trithiane (No. 574) (Mondino et al., 1981) is at least 150 000 times the estimated daily intake of methylthiomethylmercaptan when used as a flavouring agent.	See note 8	No safety concern

Table 1 (contd)

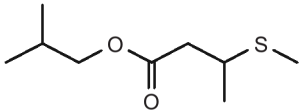
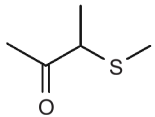
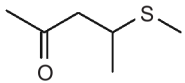
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
(±)-Isobutyl 3-methylthiobutyrate	1677	127931-21-9 	No Europe: 0.2 USA: 3 Japan: ND	Yes. The NOEL of 1.4 mg/kg bw per day for the related substance 2-(methylthiomethyl)-3-phenylpropenal (No. 505) (Cox et al., 1978) is at least 28 000 times the estimated daily intake of (±)-isobutyl 3-methylthiobutyrate when used as a flavouring agent.	See notes 1 and 2	No safety concern
3-(Methylthio)-2-butanone	1688	53475-15-3 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is >3 million times the estimated daily intake of 3-(methylthio)-2-butanone when used as a flavouring agent.	See notes 1 and 4	No safety concern
4-(Methylthio)-2-pentanone	1689	143764-28-7 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 1.9 mg/kg bw per day for the related substance 3-mercapto-2-pentanone (No. 560) (Morgareidge, 1971) is >9 million times the estimated daily intake of 4-(methylthio)-2-pentanone when used as a flavouring agent.	See notes 1 and 4	No safety concern

Table 1 (contd)

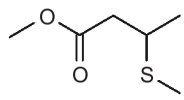
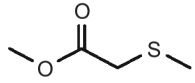
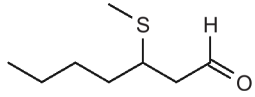
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Methyl 3-(methylthio)-butanoate	1690	207983-28-6 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >32 million times the estimated daily intake of methyl 3-(methylthio)-butanoate when used as a flavouring agent.	See notes 1 and 2	No safety concern
Methyl (methylthio)-acetate	1691	16630-66-3 	No Europe: 0.1 USA: 1 Japan: 0.1	Yes. The NOEL of 1.4 mg/kg bw per day for the related substance 2-(methylthiomethyl)-3-phenylpropenal (No. 505) (Cox et al., 1978) is at least 70 000 times the estimated daily intake of methyl (methylthio)acetate when used as a flavouring agent.	See notes 1 and 2	No safety concern
(±)-3-(Methylthio)-heptanal	1692	51755-70-5 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 1.4 mg/kg bw per day for the related substance 2-(methylthiomethyl)-3-phenylpropenal (No. 505) (Cox et al., 1978) is 7 million times the estimated daily intake of (±)-3-(methylthio)heptanal when used as a flavouring agent.	See notes 1 and 5	No safety concern

Table 1 (contd)

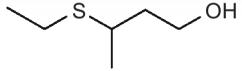
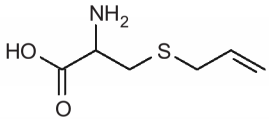
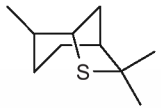
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
(±)-3-(Ethylthio)butanol	1703	117013-33-9 	No Europe: ND USA: 0.1 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is 350 000 times the estimated daily intake of (±)-3-(ethylthio)butanol when used as a flavouring agent.	See notes 1 and 3	No safety concern
Structural class III						
S-Allyl-L-cysteine	1710	21593-77-1 	No Europe: ND USA: 2 Japan: ND	Yes. The NOEL of 250 mg/kg bw per day (Kodera et al., 2002) is >8 million times the estimated daily intake of *S*-allyl-L-cysteine when used as a flavouring agent.	See notes 1 and 2	No safety concern
Heterocyclic sulfides						
Structural class I						
(±)-2,8-Epithio-*cis*-*p*-menthane	1685	68398-18-5 	No Europe: 0.4 USA: ND Japan: 0.03	Yes. The NOEL of 10 mg/kg bw per day (Finlay, 2004) is >1 million times the estimated daily intake of (±)-2,8-epithio-*cis*-*p*-menthane when used as a flavouring agent.	See note 6	No safety concern

Table 1 (contd)

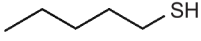
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Simple thiols						
Structural class I						
Ethanethiol	1659	75-08-1 	No Europe: 0.4 USA: ND Japan: 0.05	Yes. The NOEL of 0.56 mg/kg bw per day for the related substance cyclopentanethiol (No. 516) (Morgareidge & Oser, 1970a) is at least 80 000 times the estimated daily intake of ethanethiol when used as a flavouring agent.	See note 8	No safety concern
1-Pentanethiol	1662	110-66-7 	No Europe: 0.05 USA: 0.2 Japan: 0.05	Yes. The NOEL of 0.56 mg/kg bw per day for the related substance cyclopentanethiol (No. 516) (Morgareidge & Oser, 1970a) is >100 000 times the estimated daily intake of 1-pentanethiol when used as a flavouring agent.	See note 8	No safety concern

Table 1 (contd)

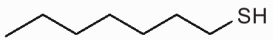
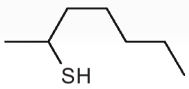
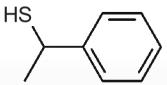
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
1-Heptanethiol	1663	1639-09-4 	No Europe: 0.03 USA: ND Japan: ND	Yes. The NOEL of 0.56 mg/kg bw per day for the related substance cyclopentanethiol (No. 516) (Morgareidge & Oser, 1970a) is >1 million times the estimated daily intake of 1-heptanethiol when used as a flavouring agent.	See note 8	No safety concern
2-Heptanethiol	1664	628-00-2 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 0.56 mg/kg bw per day for the related substance cyclopentanethiol (No. 516) (Morgareidge & Oser, 1970a) is >2 million times the estimated daily intake of 2-heptanethiol when used as a flavouring agent.	See note 8	No safety concern
Structural class II						
(±)-1-Phenylethyl-mercaptan	1665	6263-65-6 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 0.43 mg/kg bw per day for the related substance 2,6-dimethylthiophenol (No. 530) (Peano et al., 1981) is >2 million times the estimated daily intake of (±)-1-phenylethylmercaptan when used as a flavouring agent.	See note 8	No safety concern

Table 1 (contd)

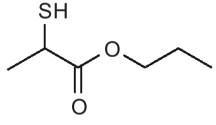
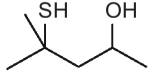
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Thiols with oxidized side-chains						
Structural class I						
Propyl 2-mercaptopropionate	1667	19788-50-2 	No Europe: 0.01 USA: 0.1 Japan: 0.03	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is at least 350 000 times the estimated daily intake of propyl 2-mercaptopropionate when used as a flavouring agent.	See notes 2 and 8	No safety concern
(±)-4-Mercapto-4-methyl-2-pentanol	1669	31539-84-1 	No Europe: 0.01 USA: 0.1 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is at least 350 000 times the estimated daily intake of (±)-4-mercapto-4-methyl-2-pentanol when used as a flavouring agent.	See notes 3 and 8	No safety concern

Table 1 (contd)

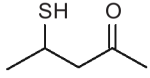
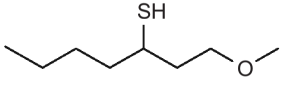
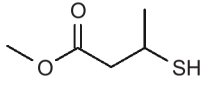
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
4-Mercapto-2-pentanone	1670	92585-08-5 	No Europe: ND USA: 0.07 Japan: ND	Yes. The NOEL of 1.9 mg/kg bw per day for the related substance 3-mercapto-2-pentanone (No. 560) (Morgareidge, 1971) is >1 million times the estimated daily intake of 4-mercapto-2-pentanone when used as a flavouring agent.	See notes 4 and 8	No safety concern
(*S*)-1-Methoxy-3-heptanethiol	1671	400052-49-5 	No Europe: 0.01 USA: 2 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is >23 000 times the estimated daily intake of (*S*)-1-methoxy-3-heptanethiol when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern
Methyl 3-mercaptobutanoate	1674	54051-19-3 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is >3 million times the estimated daily intake of methyl 3-mercaptobutanoate when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern

Table 1 (contd)

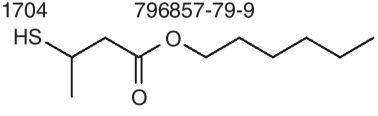
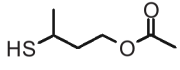
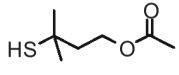
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Hexyl 3-mercaptobutanoate	1704	796857-79-9 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is >3 million times the estimated daily intake of hexyl 3-mercaptobutanoate when used as a flavouring agent.	See notes 2 and 8	No safety concern
(±)-3-Mercapto-1-butyl acetate	1705	89534-38-3 	No Europe: ND USA: 0.1 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is 350 000 times the estimated daily intake of (±)-3-mercapto-1-butyl acetate when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern
3-Mercapto-3-methyl-1-butyl acetate	1706	50746-09-3 	No Europe: ND USA: 0.1 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is 350 000 times the estimated daily intake of 3-mercapto-3-methyl-1-butyl acetate when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern

Table 1 (contd)

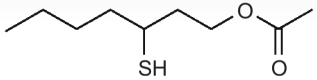
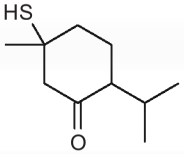
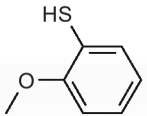
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
3-Mercaptoheptyl acetate	1708	548774-80-7 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is >3 million times the estimated daily intake of 3-mercaptoheptyl acetate when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern
Structural class II						
cis- and *trans*-Mercapto-*p*-menthan-3-one	1673	29725-66-4 	No Europe: 1 USA: ND Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2-mercapto-3-butanol (No. 546) (Cox et al., 1974) is 35 000 times the estimated daily intake of *cis*- and *trans*-mercapto-*p*-menthan-3-one when used as a flavouring agent.	See notes 4 and 8	No safety concern
Structural class III						
2-Mercaptoanisole	1666	7217-59-6 	No Europe: 1 USA: ND Japan: 0.03	The NOEL of 0.51 mg/kg bw per day for the related substance 2-mercaptomethylbenzene (No. 528) (Posternak et al., 1969) is at least 25 500 times the estimated daily intake of 2-mercaptoanisole when used as a flavouring agent.	See notes 2, 3 and 8	No safety concern

Table 1 (contd)

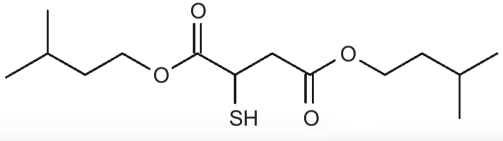
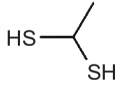
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Diisopentyl thiomalate	1672	68084-03-7 	No Europe: 0.01 USA: ND Japan: ND	No, proceeded to step B5	See notes 2, 3 and 8	No safety concern[e]
Dithiols						
Structural class I						
Ethane-1,1-dithiol	1660	69382-62-3 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOELs of 125 mg/kg bw per day and 6.5 mg/kg bw per day for the hydrolysis products acetaldehyde (No. 80) (Til et al., 1988) and hydrogen sulfide (Chemical Industry Institute of Technology, 1983), respectively, are 625 million and >32 million times the estimated daily intake of ethane-1,1-dithiol when used as a flavouring agent.	See note 8	No safety concern

Table 1 (contd)

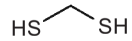
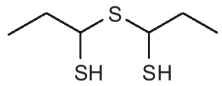
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Dimercaptomethane	1661	6725-64-0 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOELs of 15 mg/kg bw per day and 6.5 mg/kg bw per day for the hydrolysis products formaldehyde (Til et al., 1989) and hydrogen sulfide (Chemical Industry Institute of Technology, 1983), respectively, are 75 million and >32 million times the estimated daily intake of dimercaptomethane when used as a flavouring agent.	See note 8	No safety concern
bis(1-Mercapto-propyl)sulfide	1709	53897-60-2 	No Europe: ND USA: 0.6 Japan: ND	Yes. The NOEL of 0.7 mg/kg bw per day for the related substance 2,3-butanedithiol (No. 539) (Morgareidge, 1974) is 70 000 times the estimated daily intake of bis(1-mercaptopropyl)sulfide when used as a flavouring agent.	See notes 1 and 8	No safety concern

Table 1 (contd)

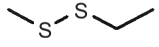
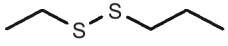
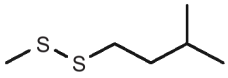
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Simple disulfides						
Structural class I						
Ethyl methyl disulfide	1693	20333-39-5 	No Europe: 0.01 USA: ND Japan: 0.03	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >14 million times the estimated daily intake of ethyl methyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Ethyl propyl disulfide	1694	30453-31-7 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >36 million times the estimated daily intake of ethyl propyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Methyl isopentyl disulfide	1696	72437-56-0 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >36 million times the estimated daily intake of methyl isopentyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern

Table 1 (contd)

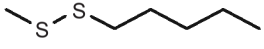
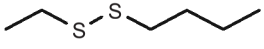
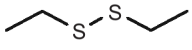
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Amyl methyl disulfide	1697	72437-68-4 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >36 million times the estimated daily intake of amyl methyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Butyl ethyl disulfide	1698	63986-03-8 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >36 million times the estimated daily intake of butyl ethyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Diethyl disulfide	1699	110-81-6 	No Europe: 0.01 USA: ND Japan: 0.03	Yes. The NOEL of 7.3 mg/kg bw per day for the related substance propyl disulfide (No. 566) (Posternak et al., 1969) is >14 million times the estimated daily intake of diethyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern

Table 1 (contd)

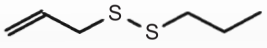
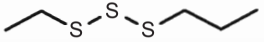
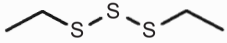
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class II						
Allyl propyl disulfide	1700	2179-59-1 	No Europe: 0.03 USA: ND Japan: 0.08	Yes. The NOEL of 4.6 mg/kg bw per day for the related substance diallyl trisulfide (No. 587) (Morgareidge & Oser, 1970b) is >4 million times the estimated daily intake of allyl propyl disulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Trisulfides						
Structural class I						
Ethyl propyl trisulfide	1695	31499-70-4 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 4.8 mg/kg bw per day for the related substance dipropyl trisulfide (No. 585) (Morgareidge & Oser, 1970c) is 24 million times the estimated daily intake of ethyl propyl trisulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern
Diethyl trisulfide	1701	3600-24-6 	No Europe: 14 USA: ND Japan: ND	Yes. The NOEL of 4.8 mg/kg bw per day for the related substance dipropyl trisulfide (No. 585) (Morgareidge & Oser, 1970c) is 24 000 times the estimated daily intake of diethyl trisulfide when used as a flavouring agent.	See notes 9 and 10	No safety concern

Table 1 (contd)

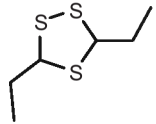
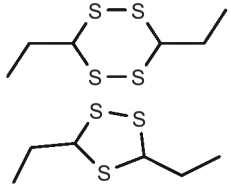
Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Heterocyclic disulfides						
Structural class II						
3,5-Diethyl-1,2,4-trithiolane	1686	54644-28-9 	No Europe: 0.6 USA: 0.01 Japan: 0.03	Yes. The NOEL of 1.9 mg/kg bw per day for the related substance 3,5-dimethyl-1,2,4-trithiolane (No. 573) (British Industrial Biological Research Association, 1976) is at least 190 000 times the estimated daily intake of 3,5-diethyl-1,2,4-trithiolane when used as a flavouring agent.	See note 11	No safety concern
Mixture of 3,6-diethyl-1,2,4,5-tetrathiane (approximately 55%) and 3,5-diethyl-1,2,4-trithiolane (approximately 45%)	1687	54717-12-3 54644-28-9 	No Europe: 0.6 USA: ND Japan: ND	Yes. The NOEL of 0.3 mg/kg bw per day for the related substance 3-methyl-1,2,4-trithiane (No. 574) (Mondino et al., 1981) is 30 000 times the estimated daily intake of the mixture of 3,6-diethyl-1,2,4,5-tetrathiane and 3,5-diethyl-1,2,4-trithiolane when used as a flavouring agent.	See note 11	No safety concern

Table 1 (contd)

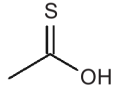
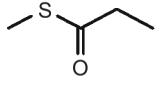
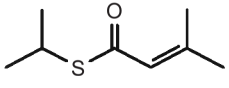
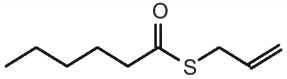
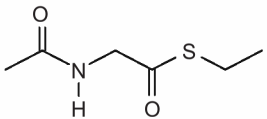
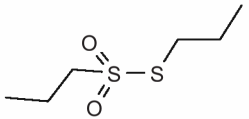
Flavouring agent	No.	CAS No. and structure	*Step B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Thioesters and acids						
Structural class I						
Thioacetic acid	1676	507-09-5 	No Europe: 0.2 USA: ND Japan: 0.4	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >900 000 times the estimated daily intake of thioacetic acid when used as a flavouring agent.	See note 12	No safety concern
S-Methyl propanethioate	1678	5925-75-7 	No Europe: 0.01 USA: 0.1 Japan: 0.03	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >3 million times the estimated daily intake of *S*-methyl propanethioate when used as a flavouring agent.	See note 12	No safety concern
S-Isopropyl 3-methylbut-2-enethioate	1679	34365-79-2 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >32 million times the estimated daily intake of *S*-isopropyl 3-methylbut-2-enethioate when used as a flavouring agent.	See note 12	No safety concern

Table 1 (contd)

Flavouring agent	No.	CAS No. and structure	*Step B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class II						
Allyl thiohexanoate	1681	156420-69-8 	No Europe: 0.01 USA: ND Japan: ND	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >32 million times the estimated daily intake of allyl thiohexanoate when used as a flavouring agent.	See note 12	No safety concern
Structural class III						
S-Ethyl 2-acetylaminoethane-thioate	1680	4396-62-7 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 6.5 mg/kg bw per day for the related substance ethyl thioacetate (No. 483) (Shellenberger, 1970) is >32 million times the estimated daily intake of *S*-ethyl 2-acetylaminoethanethioate when used as a flavouring agent.	See note 12	No safety concern
Propyl propane thiosulfonate	1702	1113-13-9 	No Europe: 0.01 USA: ND Japan: ND	No, proceeded to step 5	See note 12	No safety concern[e]

CAS, Chemical Abstracts Service; ND, no data reported.

Table 1 (contd)

[a] One hundred and forty-nine flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *143* and *166*). To facilitate the evaluations, the group was divided into 12 subgroups based on the position of the sulfur atom. The subgroup designations are indicated in the table.
[b] *Step 1*: Forty flavouring agents are in structural class I, six are in structural class II and five are in structural class III.
[c] *Step 2*: None of the agents in this group can be predicted to be metabolized to innocuous products.
[d] The thresholds for human combined per capita intake for structural classes I, II and III are 1800, 540 and 90 µg/person per day, respectively. All intake values are expressed in µg/day.
[e] *Step B*5: Conditions of use do not result in an exposure greater than 1.5 µg/day; therefore, the substance is not expected to be a safety concern.

Notes:
1.The sulfur is expected to be oxidized to the sulfoxide and sulfone.
2.The ester is expected to undergo hydrolysis to the corresponding carboxylic acid and alcohol.
3.The hydroxy group is expected to undergo oxidation to the carboxylic acid.
4.The ketone group is expected to be reduced to the alcohol, conjugated and subsequently excreted.
5.The aldehyde group is expected to be oxidized to the corresponding carboxylic acid, conjugated and subsequently excreted.
6.The sulfur is expected to be oxidized to the sulfoxide.
7.Sulfur is expected to undergo oxidative desulfuration to yield an aldehyde intermediate.
8.Sulfur is expected to be oxidized to sulfonic acid, undergo alkylation and conjugation and be excreted.
9.The di- or trisulfides are expected to be reduced to free thiols.
10. Free thiols may form mixed disulfides with glutathione or cysteine.
11. The heterocyclic disulfide is expected to undergo reduction to produce a dithiol and oxidation of the cyclic thioether.
12. The thioester is expected to undergo hydrolysis to acetate and the corresponding thiol, which will be further oxidized.

Table 2. Annual volumes of production of simple aliphatic and aromatic sulfides and thiols used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
Ethanethiol (1659)						
Europe	4	0.4	0.007			
USA	ND	ND	ND		+[e]	NA
Japan	0.2	0.05	0.0008			
Ethane-1,1-dithiol (1660)						
Europe	0.1	0.01	0.0002			
USA	0.1	0.01	0.0002		+[f]	NA
Japan	ND	ND	ND			
Dimercaptomethane (1661)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		–	NA
Japan	ND	ND	ND			
1-Pentanethiol (1662)						
Europe	0.5	0.05	0.0008			
USA	2	0.2	0.003		211[g]	106
Japan	0.2	0.05	0.0008			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
1-Heptanethiol (1663)						
Europe	0.3	0.03	0.0005			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
2-Heptanethiol (1664)						
Europe	0.1	0.01	0.0002			
USA	0.1	0.01	0.0002		+[f]	NA
Japan	ND	ND	ND			
(±)-1-Phenylethylmercaptan (1665)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[h]	NA
Japan	ND	ND	ND			
2-Mercaptoanisole (1666)						
Europe	12	1	0.02	0.005		
USA	ND	ND	ND		–	NA
Japan	0.1	0.03	0.0005	0.0001		

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
Propyl 2-mercaptopropionate (1667)						
Europe	0.1	0.01	0.0002	0.000 08		
USA	1	0.1	0.002	0.0008	+[f]	NA
Japan	0.1	0.03	0.0005	0.0002		
Methionyl butyrate (1668)						
Europe	0.1	0.01	0.0002	0.0001		
USA	ND	ND	ND		+[f]	NA
Japan	ND	ND	ND			
(±)-4-Mercapto-4-methyl-2-pentanol (1669)						
Europe	0.1	0.01	0.0002			
USA	1	0.1	0.002		–	NA
Japan	ND	ND	ND			
4-Mercapto-2-pentanone (1670)						
Europe	ND	ND	ND			
USA	0.5	0.07	0.001		–	NA
Japan	ND	ND	ND			
(*S*)-1-Methoxy-3-heptanethiol (1671)						
Europe	0.1	0.01	0.0002	0.000 04		
USA	14	2	0.03	0.006	–	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
Diisopentyl thiomalate (1672)						
Europe	0.1	0.01	0.0002	0.000 06		
USA	ND	ND	ND		–	NA
Japan	ND	ND	ND			
cis- and *trans*-Mercapto-*p*-menthan-3-one (1673)						
Europe	13	1	0.02			
USA	ND	ND	ND		–	NA
Japan	ND	ND	ND			
Methyl 3-mercaptobutanoate (1674)						
Europe	0.1	0.01	0.0002	0.000 05		
USA	0.1	0.01	0.0002	0.000 05	+[f]	NA
Japan	ND	ND	ND			
Methylthiomethylmercaptan (1675)						
Europe	0.1	0.01	0.0002			
USA	1	0.1	0.002		–	NA
Japan	ND	ND	ND			
Thioacetic acid (1676)						
Europe	2	0.2	0.003			
USA	ND	ND	ND		+[f]	NA
Japan	1	0.4	0.007			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
(±)-Isobutyl 3-methylthiobutyrate (1677)						
Europe	2	0.2	0.003	0.001		
USA	25	3	0.05	0.02	+[f]	NA
Japan	ND	ND	ND			
S-Methyl propanethioate (1678)						
Europe	0.1	0.01	0.0002			
USA	1	0.1	0.002		+	NA
Japan	0.1	0.03	0.0005			
S-Isopropyl 3-methylbut-2-enethioate (1679)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		–	NA
Japan	ND	ND	ND			
S-Ethyl 2-acetylaminoethanethioate (1680)						
Europe	0.1	0.01	0.0002			
USA	0.1	0.01	0.0002		+[f]	NA
Japan	ND	ND	ND			
Allyl thiohexanoate (1681)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		–	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
2-Methyl-1-methylthio-2-butene (1683)						
Europe	0.1	0.01	0.0002			
USA	1	0.1	0.002		+[f]	NA
Japan	ND	ND	ND			
2,4,6-Trithiaheptane (1684)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[f]	NA
Japan	ND	ND	ND			
(±)-2,8-Epithio-*cis*-*p*-menthane (1685)						
Europe	4	0.4	0.007			
USA	ND	ND	ND		+[i]	NA
Japan	0.1	0.03	0.0005			
3,5-Diethyl-1,2,4-trithiolane (1686)						
Europe	5	0.6	0.01			
USA	0.1	0.01	0.0002		+[e]	NA
Japan	0.1	0.03	0.0005			
Mixture of 3,6-diethyl-1,2,4,5-tetrathiane and 3,5-diethyl-1,2,4-trithiolane (1687)						
Europe	5	0.6	0.01			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
3-(Methylthio)-2-butanone (1688)						
Europe	0.1	0.01	0.0002			
USA	0.1	0.01	0.0002		–	NA
Japan	ND	ND	ND			
4-(Methylthio)-2-pentanone (1689)						
Europe	0.1	0.01	0.0002			
USA	0.1	0.01	0.0002		–	NA
Japan	ND	ND	ND			
Methyl 3-(methylthio)butanoate (1690)						
Europe	0.1	0.01	0.0002	0.000 04		
USA	0.1	0.01	0.0002	0.000 04	+[f]	NA
Japan	ND	ND	ND			
Methyl (methylthio)acetate (1691)						
Europe	1	0.1	0.002	0.0005		
USA	8	1	0.02	0.005	292[g]	37
Japan	0.4	0.1	0.002	0.0005		
(±)-3-(Methylthio)heptanal (1692)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
Ethyl methyl disulfide (1693)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	0.1	0.03	0.0005			
Ethyl propyl disulfide (1694)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
Ethyl propyl trisulfide (1695)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
Methyl isopentyl disulfide (1696)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
Amyl methyl disulfide (1697)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
Butyl ethyl disulfide (1698)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
Diethyl disulfide (1699)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[j]	NA
Japan	0.1	0.03	0.0005			
Allyl propyl disulfide (1700)						
Europe	0.3	0.03	0.0005			
USA	ND	ND	ND		+[k]	NA
Japan	0.3	0.08	0.001			
Diethyl trisulfide (1701)						
Europe	131	14	0.2			
USA	ND	ND	ND		+[e]	NA
Japan	ND	ND	ND			
Propyl propane thiosulfanate (1702)						
Europe	0.1	0.01	0.0002			
USA	ND	ND	ND		+[l]	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
(±)-3-(Ethylthio)butanol (1703)						
Europe	ND	ND	ND			
USA	1	0.1	0.002		+[e]	NA
Japan	ND	ND	ND			
Hexyl 3-mercaptobutanoate (1704)						
Europe	0.1	0.01	0.0002	0.0001		
USA	0.1	0.01	0.0002	0.0001	+[f]	NA
Japan	ND	ND	ND			
(±)-3-Mercapto-1-butyl acetate (1705)						
Europe	ND	ND	ND			
USA	1	0.1	0.002		–	NA
Japan	ND	ND	ND			
3-Mercapto-3-methyl-1-butyl acetate (1706)						
Europe	ND	ND	ND			
USA	1	0.1	0.002		+[m]	NA
Japan	ND	ND	ND			
2,5-Dithiahexane (1707)						
Europe	ND	ND	ND			
USA	1	0.1	0.002		+[f]	NA
Japan	ND	ND	ND			

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Annual volume in naturally occurring foods (kg)	Consumption ratio[d]
		μg/day	μg/kg bw per day			
3-Mercaptoheptyl acetate (1708)						
Europe	0.1	0.01	0.0002	0.0002		
USA	0.1	0.01	0.0002		–	NA
Japan	ND	ND	ND			
bis(1-Mercaptopropyl)sulfide (1709)						
Europe	ND	ND	ND			
USA	5	0.6	0.01		+[f]	NA
Japan	ND	ND	ND			
S-Allyl-L-cysteine (1710)						
Europe	ND	ND	ND			
USA	15	2	0.03		+[n]	NA
Japan	ND	ND	ND			
Total						
Europe	182					
USA	80					
Japan	3					

bw, body weight; NA, not applicable; ND, no data reported; +, reported to occur naturally in foods (Nijssen et al., 2006), but no quantitative data; –, not reported to occur naturally in foods.

[a] From European Flavour and Fragrance Association (2005); Flavor and Extract Manufacturers Association (2006); and Japanese Flavor and Fragrance Manufacturers Association (2002). Total poundage values of <1 kg reported in the surveys (Japanese Flavor and Fragrance Manufacturers Association,

Table 2 (contd)

2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006) have been truncated to one place following the decimal point (0.1 kg).

[b] Intake (μg/person per day) calculated as follows:
[(annual volume, kg) × (1 × 10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = 32 × 10^6 for Europe, 28 × 10^6 for the USA and 13 × 10^6 for Japan; and where correction factor = 0.8 for surveys in Europe, the USA and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006).
Intake (μg/kg bw per day) calculated as follows:
(μg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur as a result of rounding.

[c] Calculated as follows: (molecular weight of alcohol/molecular weight of ester) × daily per capita intake ("consumers only") of ester.

[d] The consumption ratio is calculated as follows:
(annual consumption via food, kg)/(most recent reported volume as a flavouring substance, kg)

[e] Nijssen et al. (2006).

[f] Natural occurrence data reported in a private communication to Flavor and Extract Manufacturers Association (2006).

[g] The annual volume in naturally occurring foods was calculated based on the annual per capita food consumption published in Stofberg & Grundschober (1987) and the published concentration in food in Nijssen et al. (2006).

[h] Kumazawa et al. (1998).

[i] Demole et al. (1982).

[j] Ishikawa et al. (1978).

[k] Boelens et al. (1971); Tada et al. (1988).

[l] Boelens et al. (1971); Freeman & Whenham (1976); Ishikawa et al. (1978); Tada et al. (1988); Kuo & Ho (1992).

[m] Tominaga & Dubourdieu (2000); Kumazawa & Masuda (2003).

[n] Kodera et al. (2002).

β-ketone (Nos 1688 and 1689) or ester (Nos 1668, 1677, 1690 and 1691), provides additional sites for biotransformation of sulfides, and the presence of these polar sites would result in increased renal excretion of these substances. The biotransformation of such oxygenated groups is well characterized and has been described for groups of flavouring agents evaluated previously by the Committee (Annex 1, references *131*, *132*, *138* and *144*). Simultaneous metabolism of sulfur and oxygenated functional groups has been reported for various substrates (Gachon et al., 1988; Karim et al., 1988; Feng & Solsten, 1991; Wilson et al., 1991; Black et al., 1993). Sulfoxide formation is usually the predominant metabolic detoxication pathway for sulfides.

1.3.3 Heterocyclic sulfides (No. 1685)

Methyl-substituted cyclic sulfides can be expected to undergo oxidation by cytochrome P450 enzymes to produce the corresponding sulfoxides (Takata et al., 1983). The mono-sulfoxides are predicted to be the main urinary metabolites of simple cyclic sulfides.

1.3.4 Thiols (Nos 1659 and 1662–1665)

Thiols are highly reactive in vivo, which is mainly due to the fact that most thiols exist in the ionized form at physiological pH. The biotransformation pathways of thiols include oxidation to unstable sulfenic acid (RSOH), which may be oxidized to the corresponding sulfinic acid (RSO_2H) and sulfonic acid (RSO_3H); methylation to yield methyl sulfides, which can be oxidized to methyl sulfoxides and sulfones; reaction with endogenous thiols such as glutathione (GSH) and cysteine to form mixed disulfides; conjugation with glucuronic acid; and oxidation of the α-carbon, which results in desulfuration and the formation of an aldehyde intermediate (McBain & Menn, 1969; Dutton & Illing, 1972; Maiorino et al., 1989; Richardson et al., 1991). There are several possible thiol–disulfide exchange reactions that may occur, and they all result from nucleophilic substitution (Cotgreave et al., 1989).

1.3.5 Thiols with oxidized side-chains (Nos 1666, 1667, 1669–1674, 1704–1706 and 1708)

The metabolism of thiols with oxidized side-chains is predicted to involve a combination of pathways described above for simple thiols, together with further oxidation or conjugation of the oxidized side-chain.

1.3.6 Dithiols (Nos 1660, 1661 and 1709)

Although they are more stable than hydrates, simple geminal dithiols (Nos 1660 and 1661) can undergo hydrolysis to yield their parent aldehydes and to release hydrogen sulfide (Mayer et al., 1963). The metabolism of the other simple aliphatic dithiol (No. 1709) is predicted to involve the pathways described above for simple thiols. Urinary metabolites could result from methylation, *S*-oxidation of a sulfur atom to yield a polar sulfonate and the formation of mixed disulfides by combination with a low molecular weight endogenous thiol such as cysteine.

1.3.7 Simple disulfides (Nos 1693, 1694 and 1696–1700)

The reduction of xenobiotic disulfides is believed to be extensive and can be catalysed enzymatically by GSH reductase (Waring, 1996) or thioltransferases (Wells et al., 1993), as well as chemically by exchange with GSH, thioredoxin, cysteine or other endogenous thiols. Reduction of non-cyclic disulfides results in the formation of thiols of low molecular weight that are metabolized via the various pathways described above for simple thiols.

1.3.8 Trisulfides (Nos 1695 and 1701)

Trisulfides are predicted to be converted rapidly to the corresponding disulfides with subsequent reduction to thiols (Moutiez et al., 1994), which are then metabolized via the various pathways described above for simple thiols.

1.3.9 Heterocyclic disulfides (Nos 1686 and 1687)

Heterocyclic disulfides are five- and six-carbon rings, which may also contain a cyclic thioether bond. The principal metabolic pathways are predicted to be disulfide reduction with ring opening to produce a dithiol and *S*-oxidation of the cyclic thioether.

1.3.10 Thioesters and acids (Nos 1676, 1678–1681 and 1702)

Thioesters are hydrolysed by lipases and esterases (Kurooka et al., 1976); the rate of hydrolysis increases as the length of the carbon chain increases and decreases as the oxygenation of the carbon chain in the thiol moiety increases (Greenzaid & Jenks, 1971). After hydrolysis, the resulting alcohol and carboxylic acid would participate in the metabolic pathways described above for sulfides containing oxygenated functional groups.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to these 51 flavouring agents, the Committee assigned 40 (Nos 1659–1664, 1667–1671, 1674–1679, 1683–1685, 1688–1699, 1701 and 1703–1709) to structural class I and 6 (Nos 1665, 1673, 1681, 1686, 1687 and 1700) to structural class II. The remaining 5 flavouring agents (Nos 1666, 1672, 1680, 1702 and 1710) were assigned to structural class III (Cramer et al., 1978).

Step 2. None of the flavouring agents in this group can be predicted to be metabolized to innocuous products. The evaluation of these substances therefore proceeded via the B-side of the Procedure.

Step B3. The estimated daily per capita intakes of the 40 flavouring agents in this group in structural class I are below the threshold of concern (i.e. 1800 µg/person per day for class I). The estimated daily per capita

intakes of the 6 flavouring agents in structural class II are below the threshold of concern (i.e. 540 µg/person per day for class II). The estimated daily per capita intakes of the 5 flavouring agents in structural class III are below the threshold of concern (i.e. 90 µg/person per day for class III). Accordingly, the evaluation of all 51 substances in the group proceeded to Step B4.

Step B4. For 2-methyl-1-methylthio-2-butene (No. 1683), the no-observed-effect level (NOEL) of 250 mg/kg body weight (bw) per day for the structurally related substance methyl sulfide (No. 452) from a 98-day study in male and female rats (Butterworth et al., 1975) provides an adequate margin of safety (at least 125 million) in relation to currently estimated levels of intake of this substance from its use as a flavouring substance. This NOEL is also appropriate for the structurally related substances 2,4,6-trithiaheptane (No. 1684) and 2,5-dithiahexane (No. 1707), because they are all simple sulfides that are anticipated to undergo oxidation and subsequent metabolism via similar metabolic pathways. In relation to the currently estimated levels of intake from use as flavouring substances, the NOEL of 250 mg/kg bw per day provides adequate margins of safety of >1 billion[3] and 125 million for 2,4,6-trithiaheptane (No. 1684) and 2,5-dithiahexane (No. 1707), respectively.

For methionyl butyrate (No. 1668), the NOEL of 1.4 mg/kg bw per day for the structurally related substance 2-(methylthiomethyl)-3-phenylpropenal (No. 505) from a 92-day study in male rats (Cox et al., 1978) provides an adequate margin of safety (7 million) in relation to currently estimated levels of intake of this substance from its use as a flavouring substance. This NOEL is also appropriate for the structurally related substances (±)-isobutyl 3-methylthiobutyrate (No. 1677), methyl (methylthio)acetate (No. 1691) and (±)-3-(methylthio)heptanal (No. 1692), because they are all acyclic sulfides with oxidized side-chains. For these structurally related substances, the NOEL of 1.4 mg/kg bw per day provides adequate margins of safety in the range of 28 000 to 7 million in relation to the currently estimated levels of intake from use as flavouring agents.

For methylthiomethylmercaptan (No. 1675), the NOEL of 0.3 mg/kg bw per day for the structurally related substance 3-methyl-1,2,4-trithiane (No. 574) from a 90-day study in rats (Mondino et al., 1981) provides an adequate margin of safety (at least 150 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For 3-(methylthio)-2-butanone (No. 1688) and (±)-3-(ethylthio)butanol (No. 1703), the NOEL of 0.7 mg/kg bw per day for the structurally related substance 2-mercapto-3-butanol (No. 546) from a 90-day

[3] Note that billion is defined as a thousand million (10^9).

study in rats (Cox et al., 1974) provides adequate margins of safety (>3 million and 350 000, respectively) in relation to estimated levels of intake of these substances from their use as flavouring agents.

For 4-(methylthio)-2-pentanone (No. 1689), the NOEL of 1.9 mg/kg bw per day for the structurally related substance 3-mercapto-2-pentanone (No. 560) from a 90-day study in rats (Morgareidge, 1971) provides an adequate margin of safety (>9 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For methyl 3-(methylthio)butanoate (No. 1690), the NOEL of 6.5 mg/kg bw per day for the structurally related substance ethyl thioacetate (No. 483) from a 91-day study in rats (Shellenberger, 1970) provides an adequate margin of safety (>32 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For *S*-allyl-L-cysteine (No. 1710), the NOEL of 250 mg/kg bw per day from a 28-day study in rats (Kodera et al., 2002) provides an adequate margin of safety (>8 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For (±)-2,8-epithio-*cis*-*p*-menthane (No. 1685), the NOEL of 10 mg/kg bw per day in female rats from a 28-day study (Finlay, 2004) provides an adequate margin of safety (>1 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For ethanethiol (No. 1659), the NOEL of 0.56 mg/kg bw per day for the structurally related substance cyclopentanethiol (No. 516) from a 90-day study in male and female rats (Morgareidge & Oser, 1970a) provides an adequate margin of safety (at least 80 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent. This NOEL is also appropriate for the structurally related substances 1-pentanethiol (No. 1662), 1-heptanethiol (No. 1663) and 2-heptanethiol (No. 1664), because they are all simple thiols. For these structurally related substances, the NOEL of 0.56 mg/kg bw per day provides adequate margins of safety in the range of >100 000 to >2 million in relation to the currently estimated levels of intake from use as flavouring agents.

For (±)-1-phenylethylmercaptan (No. 1665), the NOEL of 0.43 mg/kg bw per day for the structurally related substance 2,6-dimethylthiophenol (No. 530) from a 90-day study in rats (Peano et al., 1981) provides an adequate margin of safety (>2 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For propyl 2-mercaptopropionate (No. 1667), the NOEL of 0.7 mg/kg bw per day for the structurally related substance 2-mercapto-3-butanol (No. 546) from a 90-day study in rats (Cox et al., 1974) provides an

adequate margin of safety (at least 350 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent. This NOEL is also appropriate for the structurally related substances (±)-4-mercapto-4-methyl-2-pentanol (No. 1669), (*S*)-1-methoxy-3-heptanethiol (No. 1671), methyl 3-mercaptobutanoate (No. 1674), hexyl 3-mercaptobutanoate (No. 1704), (±)-3-mercapto-1-butyl acetate (No. 1705), 3-mercapto-3-methyl-1-butyl acetate (No. 1706), 3-mercaptoheptyl acetate (No. 1708) and *cis*- and *trans*-mercapto-*p*-menthan-3-one (No. 1673), because they are all thiols with oxidized side-chains. For these structurally related substances, the NOEL of 0.7 mg/kg bw per day provides adequate margins of safety in the range of >23 000 to >3 million in relation to the currently estimated intakes from use as flavouring agents.

For 4-mercapto-2-pentanone (No. 1670), the NOEL of 1.9 mg/kg bw per day for the structurally related substance 3-mercapto-2-pentanone (No. 560) from a 90-day study in rats (Morgareidge, 1971) provides an adequate margin of safety (>1 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For 2-mercaptoanisole (No. 1666), the NOEL of 0.51 mg/kg bw per day for the structurally related substance 2-mercaptomethylbenzene (No. 528) from a 90-day study in rats (Posternak et al., 1969) provides an adequate margin of safety (at least 25 500) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For ethane-1,1-dithiol (No. 1660), the NOEL of 125 mg/kg bw per day for one hydrolysis product, acetaldehyde (No. 80), from a 28-day study in rats (Til et al., 1988) and the NOEL of 6.5 mg/kg bw per day for the other hydrolysis product, hydrogen sulfide, from a 90-day inhalation study in rats (Chemical Industry Institute of Technology, 1983) provide adequate margins of safety (625 million and >32 million, respectively) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For dimercaptomethane (No. 1661), the NOEL of 15 mg/kg bw per day for one hydrolysis product, formaldehyde, from a 2-year study in rats (Til et al., 1989) and the NOEL of 6.5 mg/kg bw per day for the other hydrolysis product, hydrogen sulfide, from a 90-day inhalation study in rats (Chemical Industry Institute of Technology, 1983) provide adequate margins of safety (75 million and >32 million, respectively) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For bis(1-mercaptopropyl)sulfide (No. 1709), the NOEL of 0.7 mg/kg bw per day for the structurally related substance 2,3-butanedithiol (No. 539) from a 90-day study in rats (Morgareidge, 1974) provides an adequate margin of safety (70 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For ethyl methyl disulfide (No. 1693), the NOEL of 7.3 mg/kg bw per day for the structurally related substance propyl disulfide (No. 566) from a 90-day study in rats (Posternak et al., 1969) provides an adequate margin of safety (>14 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent. This NOEL is also appropriate for the structurally related substances ethyl propyl disulfide (No. 1694), methyl isopentyl disulfide (No. 1696), amyl methyl disulfide (No. 1697), butyl ethyl disulfide (No. 1698) and diethyl disulfide (No. 1699), because they are all simple disulfides. For these structurally related substances, the NOEL of 7.3 mg/kg bw per day provides adequate margins of safety in the range of >14 million to >36 million in relation to the currently estimated intakes of these substances from use as flavouring agents.

For allyl propyl disulfide (No. 1700), the NOEL of 4.6 mg/kg bw per day for the structurally related substance diallyl trisulfide (No. 587) from a 90-day study in rats (Morgareidge & Oser, 1970b) provides an adequate margin of safety (>4 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For ethyl propyl trisulfide (No. 1695), the NOEL of 4.8 mg/kg bw per day for the structurally related substance dipropyl trisulfide (No. 585) from a 90-day study in rats (Morgareidge & Oser, 1970c) provides an adequate margin of safety (24 million) in relation to currently estimated levels of intake of this substance from use as a flavouring agent. This NOEL is also appropriate for the structurally related substance diethyl trisulfide (No. 1701), because it is also a trisulfide. The NOEL of 4.8 mg/kg bw per day provides an adequate margin of safety of 24 000 for this substance in relation to the currently estimated level of intake from use as a flavouring agent.

For 3,5-diethyl-1,2,4-trithiolane (No. 1686), the NOEL of 1.9 mg/kg bw per day for the structurally related substance 3,5-dimethyl-1,2,4-trithiolane (No. 573) from a 91-day study in rats (British Industrial Biological Research Association, 1976) provides an adequate margin of safety (at least 190 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For the mixture of 3,6-diethyl-1,2,4,5-tetrathiane (approximately 55%) and 3,5-diethyl-1,2,4-trithiolane (approximately 45%) (No. 1687), the NOEL of 0.3 mg/kg bw per day for the structurally related substance 3-methyl-1,2,4-trithiane (No. 574) from a 90-day study in rats (Mondino et al., 1981) provides an adequate margin of safety (30 000) in relation to currently estimated levels of intake of this substance from use as a flavouring agent.

For thioacetic acid (No. 1676), the NOEL of 6.5 mg/kg bw per day for the structurally related substance ethyl thioacetate (No. 483) from a 91-day study in rats (Shellenberger, 1970) provides an adequate margin of safety (>900 000) in relation to currently estimated levels of

intake of this substance from use as a flavouring agent. This NOEL is also appropriate for the structurally related substances *S*-methyl propanethioate (No. 1678), *S*-isopropyl 3-methylbut-2-enethioate (No. 1679), allyl thiohexanoate (No. 1681) and *S*-ethyl 2-acetylaminoethanethioate (No. 1680), because they are all thioesters and related acids. For these structurally related substances, the NOEL of 6.5 mg/kg bw per day provides adequate margins of safety in the range of >3 million to >32 million in relation to their currently estimated levels of intake from their use as flavouring agents.

Step B5. Two substances, diisopentyl thiomalate (No. 1672) and propyl propane thiosulfonate (No. 1702), were evaluated at this step of the Procedure. The currently estimated daily per capita intakes of both substances are below 1.5 µg/person per day in Europe. Applying the criteria for Step B5 outlined in Annex 5 of the evaluations published after its forty-ninth meeting (Annex 1, reference *131*), the Committee concluded that the use of these substances as flavouring agents at their currently estimated levels of intake poses no safety concern.

Table 1 summarizes the evaluations of the 51 simple aliphatic and aromatic sulfides and thiols in this group.

1.5 Consideration of combined intakes from use as flavouring agents

The substances in this group that have been evaluated at this meeting and at the fifty-third and sixty-first meetings are predicted to be metabolized by a variety of metabolic pathways. Because of the diverse structures, there are few common metabolites. Examples are 3-(methylthio)propionic acid (from Nos 476 and 468) and thioacetic acid (from Nos 482, 483, 485 and 491). The combined intakes of substances with a common metabolite were below the relevant threshold of toxicological concern (TTC) value.

Several substances in this group contain a sulfide group, which would be metabolized by *S*-oxidation. These include simple sulfides and sulfoxides for which *S*-oxidation would be the main route of metabolism (Nos 452–460, 507, 533, 1683, 1684, 1685 and 1707), acyclic sulfides with oxidized side-chains, which would have alternative processes of elimination (Nos 461–463, 465–481, 495–503, 505, 1297, 1298, 1668, 1677, 1688–1692, 1703 and 1710), and cyclic sulfides, some of which have additional functional groups, providing alternative processes of elimination (Nos 456, 464, 498, 499, 534, 543, 550, 563, 1296 and 1685). The majority of the combined intake of all compounds was from methyl sulfide (No. 452). The Committee concluded that under the current conditions of use as flavouring agents, the combined intake of these substances would not saturate *S*-oxidation and combined intakes would not raise safety concerns.

A number of substances in this group contain a thiol group, which is predicted to be metabolized by methylation followed by oxidation, conjugation with GSH, *S*-glucuronidation and/or oxidation to sulfonic acids. These would be the major routes

of elimination of simple alkyl and aryl thiols (Nos 508–531, 1659 and 1662–1665) and dithiols (Nos 532, 535–542, 1660, 1661 and 1709), whereas alternative processes of elimination would be available for thiols with additional functional groups (Nos 544–549, 551–561, 563, 1289–1294, 1666, 1667, 1669–1675, 1704–1706 and 1708). The Committee concluded that under the current conditions of their use as flavouring agents, the combined intake of these substances would not saturate the metabolic pathways and combined intakes would not raise safety concerns.

A number of substances contain a disulfide or polysulfide group, which is predicted to be biotransformed initially by reduction to thiols, which would then be metabolized as described above. Some substances were simple alkyl and aryl disulfides or polysulfides (Nos 564–579, 582–588, 1299, 1300, 1686, 1687 and 1693–1701), whereas alternative processes of elimination would be available for disulfides or polysulfides with additional functional groups (Nos 580 and 581). The Committee concluded that under the current conditions of use as flavouring agents, the combined intake of these substances would not saturate the metabolic pathways and combined intakes would not raise safety concerns.

Some substances were thioic acids (No. 1676) and their esters (Nos 482–494, 504, 506, 1295 and 1678–1681), which are predicted to be eliminated by conversion to the oxy-analogue and/or excretion as the thioic acid. Thioesters (Nos 482–494, 504, 506, 1295 and 1678–1681) would be hydrolysed to the corresponding thioic acid prior to elimination. The Committee concluded that under the current conditions of use as flavouring agents, the combined intake of these substances would not saturate the metabolic pathways and combined intakes would not raise safety concerns.

1.6 Consideration of secondary components

Fourteen members of this group of flavouring substances, ethane-1,1-dithiol (No. 1660), 4-mercapto-2-pentanone (No. 1670), diisopentyl thiomalate (No. 1672), *cis*- and *trans*-mercapto-*p*-menthan-3-one (No. 1673), 2,4,6-trithiaheptane (No. 1684), (±)-2,8-epithio-*cis*-*p*-menthane (No. 1685), mixture of 3,6-diethyl-1,2,4,5-tetrathiane and 3,5-diethyl-1,2,4-trithiolane (No. 1687), (±)-3-(methylthio)heptanal (No. 1692), ethyl methyl disulfide (No. 1693), ethyl propyl trisulfide (No. 1695), methyl isopentyl disulfide (No. 1696), butyl ethyl disulfide (No. 1698), allyl propyl disulfide (No. 1700) and bis(1-mercaptopropyl)sulfide (No. 1709), have assay values of <95%. Information on the safety of the secondary components of these 14 compounds is summarized in Annex 5 (Summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%). The secondary component of diisopentyl thiomalate, diisopentyl thiotartronate, is expected to share the same metabolic fate as the primary substance. The secondary components of *trans*-mercapto-*p*-menthan-3-one, piperitone (No. 435) and α-terpineol (No. 366), are expected to undergo rapid absorption, distribution, metabolism and excretion and were evaluated at previous

meetings (Annex 1, references *137* and *166*). The secondary component of (±)-2,8-epithio-*cis*-*p*-menthane, d-limonene (No. 1326), is expected to undergo rapid absorption, distribution, metabolism and excretion and was evaluated at a previous meeting (Annex 1, reference *173*). The secondary component of (±)-3-(methylthio)-heptanal, *trans*-2-heptenal (No. 1360), is expected to undergo rapid absorption, distribution, metabolism and excretion and was evaluated at a previous meeting (Annex 1, reference *173*). The secondary components of ethyl methyl disulfide, diethyl disulfide and dimethyl disulfide (No. 564), are expected to share the same metabolic fate as ethyl methyl disulfide. Dimethyl disulfide was evaluated at a previous meeting (Annex 1, reference *160*). The secondary components of ethyl propyl trisulfide, diethyl trisulfide and dipropyl trisulfide (No. 585), are expected to share the same metabolic fate as ethyl propyl trisulfide. Dipropyl trisulfide was evaluated at a previous meeting (Annex 1, reference *149*). The secondary components of methyl isopentyl disulfide, dimethyl disulfide (No. 564) and diisopentyl disulfide, are expected to share the same metabolic fate as methyl isopropyl disulfide. Dimethyl disulfide was evaluated at a previous meeting (Annex 1, reference *160*). The secondary components of butyl ethyl disulfide, diethyl disulfide and dibutyl disulfide, are expected to share the same metabolic fate as butyl ethyl disulfide. The secondary components of allyl propyl disulfide, allyl propylsulfide and dipropylsulfide, are expected to share the same metabolic fate as allyl propyl disulfide. The secondary component of bis(1-mercaptopropyl)sulfide, 3,5-diethyl-1,2,4-trithiolane (No. 1686), was evaluated at the present meeting and is predicted to undergo reduction to free dithiol and *S*-oxidation of the cyclic thioether with subsequent excretion. Owing to their malodorous nature, the following flavouring agents are available in solution: ethane-1,1-dithiol in 1% ethanol (No. 41), 4-mercapto-2-pentanone in 1% acetoin (No. 405), 2,4,6-trithiaheptane in 10% triacetin (No. 920) and the mixture of 3,6-diethyl-1,2,4,5-tetrathiane and 3,5-diethyl-1,2,4-trithiolane in 1% vegetable oil. The first three solvents have been evaluated at previous meetings (Annex 1, references *122*, *137* and *160*), and vegetable oil is a common component of traditional foods. None of the secondary components is considered to present a safety concern at current levels of intake as flavouring agents.

1.7 Conclusion

In the previous evaluations of substances in this group, studies of acute toxicity, short-term toxicity (14 days to 14 weeks), long-term toxicity and carcinogenicity, genotoxicity and reproductive toxicity were available. None raised safety concerns. The toxicity data available for this evaluation were supported by those from the previous evaluations.

The Committee concluded that these 51 flavouring agents, which are additions to the group of simple aliphatic and aromatic sulfides and thiols evaluated previously, would not give rise to safety concerns at the currently estimated levels of intake.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of 51 flavouring agents, which are additions to the group of simple aliphatic and aromatic sulfides and thiols evaluated previously.

2.2 Additional considerations on intake

There is no additional information on intake.

2.3 Biological data

2.3.1 Biochemical data: absorption, distribution, metabolism and excretion

Additional information related to the absorption, distribution, metabolism and excretion of these agents and of structurally related agents has been reported since the submission of the original monograph (Annex 1, reference *144*) and the first addendum (Annex 1, reference *167*). These data are cited below.

(a) Simple sulfides

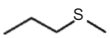
(i) Methyl propyl sulfide[4]

Studies conducted in rabbit liver microsomes on the metabolism of methyl, ethyl, isopropyl and propyl thiols show that rabbit liver catalyses the *S*-methylation of short-chain alkane thiols to yield the corresponding methyl sulfides. The coenzyme in this process is *S*-adenosyl-L-methionine. The resulting methyl sulfides are further metabolized by formation of the corresponding sulfoxide and sulfone (Holloway et al., 1979). The methylation of short-chain alkyl thiols to methylthioethers acts as a detoxication mechanism for the reactive sulfhydryl group (Holloway et al., 1979).

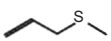
(ii) Allyl methyl sulfide[5]

Allyl methyl sulfide, allyl mercaptan and methyl mercaptan were detected in the expired air of human subjects after the subjects chewed and ate 1000 mg of grated raw or grated heat-treated garlic for 30 s. Analytical concentrations of allyl methyl sulfide in raw garlic and heated garlic at 0 min were about 0.03 mg/kg and 0.005 mg/kg, respectively, and after 30 min had decreased to approximately 0.01 mg/kg and <0.005 mg/kg, respectively (Tamaki & Sonoki, 1999).

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Metabolites in breath, plasma and simulated gastric fluids were analysed after human subjects consumed dehydrated granular garlic and an enteric-coated garlic preparation. Allicin, released by the garlic preparations, decomposed in the stomach acid or, with time, in the intestine, to release allyl sulfides, disulfides and other volatile sulfur compounds. These compounds are expected to be further metabolized by conjugation with GSH and/or *S*-adenosylmethionine-dependent methylation. Results of the studies show that the major volatile metabolite from the consumption of dehydrated granular garlic and an enteric-coated garlic preparation is allyl methyl sulfide (Rosen et al., 2000, 2001).

Allyl mercaptan and allyl methyl sulfide were identified as metabolites of diallyl disulfide when primary rat hepatocytes were incubated with either diallyl disulfide or diallyl sulfide. The highest identified concentration of allyl methyl sulfide (0.93 ± 0.08 µg/ml at 90 min) was markedly lower than that of allyl mercaptan (46.2 ± 6.6 µg/ml at 60 min). The results also showed that allyl methyl sulfide is a metabolite of diallyl sulfide (Sheen et al., 1999).

(b) Acyclic sulfides with oxidized and thiol side-chains

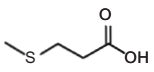
(i) S-Allyl-L-cysteine (No. 1710)

In a pharmacokinetic study, three healthy human male volunteers were given 500 mg of a garlic supplement containing *S*-allyl-L-cysteine. Blood samples were collected from the brachiocephalic vein before administration and at 1, 5 and 23 h following administration. Peak plasma concentrations of 23 ng/ml were detected 1 h after administration, indicating rapid absorption from the gastrointestinal tract and high bioavailability in humans. Results also showed that *S*-allyl-L-cysteine has a half-life of more than 10 h and a clearance time of more than 30 h in humans (Kodera et al., 2002). Recovery tests showed that 87% and 100% of *S*-allyl-L-cysteine were recovered 3 h after the addition of the compound to a red blood cell fraction and a plasma fraction, indicating that *S*-allyl-L-cysteine is stable in the blood (Kodera et al., 2002).

(ii) 3-(Methylthio)propionic acid[6]

A metabolism study conducted in rat liver homogenate showed that carbon dioxide, sulfate, methanethiol and hydrogen sulfide are intermediary or excreted metabolites of the salt of 3-(methylthio)propionic acid (Steele & Benevenga, 1979). Developmental changes in the metabolism of the salt of 3-(methylthio)propionic acid were measured in rats from day 1 to day 400. During the 1st week of life, the metabolic capacity of liver homogenates to produce methanethiol and hydrogen sulfide from 3-methyl[^{35}S]thiopropionate increased 6-fold and remained at that elevated level through weaning, then gradually decreased to the value observed in

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the 1-day-old rat by 400 days of age. This pattern does not change when the data are expressed in relation to tissue oxygen consumption, thereby indicating that the ability of young rats to produce methanethiol and hydrogen sulfide from 3-methyl[^{35}S]thiopropionate is not due solely to a higher metabolic rate (Finkelstein & Benevenga, 1984).

(c) Heterocyclic sulfides

(i) Tetrahydrothiophene[7]

In a metabolism study, six male Wistar rats were administered 20.3 mg of 1,4-dibromobutane in arachis oil via intraperitoneal injection. Urine samples were collected during the 24-h period prior to dosing and at 24 and 48 h after dosing. The only stable sulfur-containing metabolites identified, tetrahydrothiophene and the corresponding hydroxylated sulfone, 3-hydroxysulfolane, were quantified for the 0- to 24-h, 24- to 48-h and 0- to 48-h time intervals using gas–liquid chromatography equipped with a flame ionization detector. At 48 h, tetrahydrothiophene and 3-hydroxysulfolane were determined to be 5.8 ± 1.1% and 57 ± 15% of the dose of the parent compound, respectively. The authors concluded that 1,4-dibromobutane undergoes extensive metabolism via GSH conjugation, resulting in the efficient detoxication of the parent compound. The initial conjugation to GSH leads to the formation of a relatively stable cyclic sulfonium ion, *N*-acetyl-*S*-(β-alanyl) tetrahydrothiophenium salt, which is excreted to a minor extent. The major fraction decomposes in vivo to tetrahydrothiophene, which undergoes further oxidative metabolism to yield 3-hydroxysulfolane. Both metabolites are subsequently excreted in the urine (Onkenhout et al., 1986).

(d) Thiols with oxidized side-chains

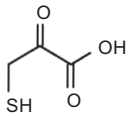
(i) 3-Mercapto-2-oxopropionic acid[8]

A metabolism study was conducted in male Wistar rats to determine the fate of the salt of 3-mercapto-2-oxopropionic acid, the intermediate product in the trans-amination pathway of L-cysteine metabolism. Results showed that the salt of 3-mercapto-2-oxopropionic acid is metabolized by reduction and *trans*-sulfuration to yield 3-mercaptolactate cysteine mixed disulfide (*S*-(2-hydroxy-2-carboxyethylthio) cysteine) and inorganic sulfate, respectively. The reduction is catalysed by lactate dehydrogenase, as indicated by the use of anti-lactate dehydrogenase antiserum.

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Formation of *S*-(2-hydroxy-2-carboxyethylthio) cysteine is favoured at low 3-mercaptopyruvate sulfurtransferase activity (Ubuka et al., 1992).

(e) Dithiols

(i) Butane-1,4-dithiol[9]

Studies show that microsomal thiol *S*-methyltransferase in rat salivary glands is highly specific to aliphatic thiols. Relative activity of 4 mmol butane-1,4-dithiol/l was 95.6% (compared with 100% for dithiothreitol). The authors concluded that microsomal thiol *S*-methyltransferase activity in rat salivary glands detoxifies extracellular thiols and intracellular hydrogen sulfide to protect normal secretory functions (Yashiro & Takatsu, 2001).

(f) Simple disulfides

(i) Diethyl disulfide (No. 1699)

Radiolabelled metabolites were identified in the urine, carcass and intestines of mice after the administration of a single subcutaneous 10 mg dose of ^{35}S-labelled diethyl disulfide (approximately 400 mg/kg bw) in an aqueous vehicle. Ethyl methyl sulfone was detected in the urine, carcass and intestines of mice after a single oral dose of 160 mg diethyl disulfide/kg bw. This product forms via reduction of diethyl disulfide to ethyl thiol, which is subsequently methylated to methyl ethyl sulfide. The sulfide is then oxidized to the corresponding sulfone. Sulfate accounted for 80–90% of the radioactivity in urine. Ethyl methyl sulfone was also detected in the urine of rabbits and guinea-pigs after single oral doses of 200 and 185 mg diethyl disulfide/kg bw, respectively (Snow, 1957).

2.2.2 Toxicological studies

(a) Acute toxicity

Oral LD_{50} values have been reported for 2 of the 51 substances evaluated in this group and are summarized in Table 3. In rats, oral LD_{50} values of 682 and 9500–10 940 mg/kg bw have been reported for ethanethiol (No. 1659) and *S*-allyl-L-cysteine (No. 1710), respectively, confirming the low acute toxicity of simple aliphatic and aromatic sulfides and thiols (Fairchild & Stokinger, 1958; Kodera et al., 2002).

In male and female mice, oral LD_{50} values of 8890 and 9390 mg/kg bw have been reported, respectively, for *S*-allyl-L-cysteine (No. 1710), which further supports the low acute toxicity of simple aliphatic and aromatic sulfides and thiols (Kodera et al., 2002).

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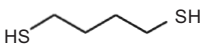


Table 3. Results of oral acute toxicity studies with simple aliphatic and aromatic sulfides and thiols used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1659	Ethanethiol	Rat; M	682	Fairchild & Stokinger (1958)
1710	*S*-Allyl-L-cysteine	Mouse; M, F	8890 (M) 9390 (F)	Kodera et al. (2002)
1710	*S*-Allyl-L-cysteine	Rat; M, F	10 940 (M) 9500 (F)	Kodera et al. (2002)

F, female; M, male.

(b) Short-term studies of toxicity

The results of two additional ~28-day studies are described below and are summarized in Table 4. The results of a 2-week diet study, a 2-year drinking-water study and a 90-day inhalation study for metabolites of ethane-1,1-dithiol (No. 1660) and dimercaptomethane (No. 1661) and a structurally related substance are also discussed below.

(i) (±)-2,8-Epithio-cis-p-*menthane (No. 1685)*

Groups of Crl:CD SD(IGS)BR rats (10 per sex per group) were administered 0 or 10 mg 2,8-epithio-*cis-p*-menthane/kg bw per day in corn oil via gavage for a period of 28 days. The animals were examined daily for overt signs of toxicity, general health and behavioural changes. Individual body weights and food consumption were recorded weekly for the first 3 weeks and on both days 27 and 28 during the last week. Haematology and blood chemistry parameter evaluations were performed on all animals at the end of the study. At necropsy, all animals were subjected to gross and microscopic examination.

There were no deaths, clinical signs of toxicity or changes in functional parameters (i.e. behavioural, functional performance and sensory reactivity) reported in any of the animals during the study. No statistically significant differences were observed in final body weights and body weight gains of treated males and females compared with control values; however, the body weight gain of treated females between days 27 and 28 was significantly greater than that observed in the control group. During the 1st week of the study, food consumption of treated females was significantly elevated compared with controls; however, mean overall food consumption and food efficiency of treated males and females did not differ significantly from controls. Haematological, coagulation and clinical chemistry parameters of animals administered (±)-2,8-epithio-*cis-p*-menthane were comparable with control values. Urinalysis revealed coarsely granular casts in all test group males. In comparison with the controls, statistically significant increases were observed in absolute kidney and liver weights and in the kidney to brain weight ratio and liver to brain weight ratio in treated females. Although final body weights of test females were not significantly different from controls, final body weights of treated

Table 4. Results of short-term studies of toxicity with simple aliphatic and aromatic sulfides and thiols used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1685	(±)-2,8-Epithio-*cis*-*p*-menthane	Rat; M, F	1/20	Oral	28	10 (F)[c]	Finlay (2004)
1710	*S*-Allyl-L-cysteine	Rat; M, F	4/20	Oral	26–28[d]	250	Kodera et al. (2002)

F, female; M, male; NOEL, no-observed-effect level.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] The NOEL applies to female rats only. A NOEL could not be determined for male rats, due to the observation of α2u-globulin male-specific nephropathy.

[d] Based on the last apparent body weight measurements in males and females.

females were 105% those of controls. As such, the increases observed in absolute kidney and liver weights and in the kidney to brain weight ratio and liver to brain weight ratio were attributable to increased final body weights. Additionally, statistically significant differences were also observed in the absolute and relative (to body) heart weights of treated females. The variations in absolute heart weights were attributed to the body weight differences, whereas the increase in relative heart weight was considered to be reflective of a marked increase in a single female. Moreover, no gross or histopathological abnormalities were observed in the female organs affected by the weight variations. Owing to the differences in body weights between the treated and control animals and the lack of gross or microscopic alterations, the increases in absolute and relative kidney, liver and heart weights in treated females were not considered to be toxicologically relevant. In males, relative (to body weight) liver weights of treated males were elevated compared with controls. The differences in the relative liver weights observed in treated males were partly attributed to increased values in two males and were not accompanied by any evidence of gross or microscopic pathology. Compared with controls, statistically significant increases were also observed in absolute and relative (to body and brain weights) kidney weights in males.

The kidney weight increases in males corresponded to focal tubular degeneration/regeneration and an increase in the number and size of hyaline droplets in the kidneys observed at histopathological examination. Necrotic nuclei and increases in eosinophilic cytoplasm were noted in some of the affected tubules. Whereas minimal to mild renal tubular regeneration was observed in 8/10 treated males, minimal tubular regeneration was observed in 4/10 controls. The tubules were often surrounded by a thin layer of fibrous connective tissue. The authors concluded that the findings were consistent with early hyaline droplet nephropathy (Finlay, 2004). A subsequent immunoassay of the kidney sections obtained from the male rats confirmed the presence of α2u-globulin in the kidneys (Swenberg &

Schoonhoven, 2004). Given that α2u-globulin is a well characterized male rat-specific phenomenon (United States Environmental Protection Agency, 1991), it is concluded that the nephropathy observed in the 28-day study has no significance for the safety evaluation of (±)-2,8-epithio-*cis*-*p*-menthane to humans. No other histopathological variations were observed in either treated male or treated female rats compared with controls.

Based on the above findings, a NOEL of 10 mg/kg bw per day was established for female rats. A NOEL could not be established for male rats in this study, as the only dose tested produced male-specific α2u-globulin nephropathy (Finlay, 2004).

(ii) S-Allyl-L-cysteine (No. 1710)

Groups of male and female Crj Wistar rats were orally administered 0, 250, 500, 1000 or 2000 mg *S*-allyl-L-cysteine/kg bw per day (10 per sex per group) via gavage for approximately 26–28 days. Animals were observed for general state and subjected to clinical pathology (i.e. haematology, biochemistry and urinalysis). At study end, animals were killed, and necropsy was conducted. Whereas body weights of low-dose males (250 mg/kg bw per day) were higher than those of the control group, reaching statistical significance beginning on day 25, male rats treated at the 1000 and 2000 mg/kg bw per day dose levels were reported to have significantly decreased body weights compared with control males from day 2 onwards. At the 500 mg/kg bw per day dose level, body weights were also significantly reduced, but only between days 2 and 18. Thereafter, body weights were comparable with controls. The reductions in male body weights were dose dependent. In females, statistically significant reductions in body weights were observed at the highest dose level (2000 mg/kg bw per day) from day 2 onwards compared with control females and at the 500 and 1000 mg/kg bw per day dose level, but only for the 1st week of the study period. Decreased body weights were accompanied by dose-dependent reductions in food consumption in both sexes. Food consumption was also reported to recover in a dose-dependent manner, with females recovering faster.

Urinalysis revealed an increase in the urinary pH values of test males compared with controls. Compared with controls, urobilinogen levels were significantly lower in males treated at the 1000 and 2000 mg/kg bw per day dose levels and in females in the 250 mg/kg bw per day dose group. Levels of red blood cells were lower in treated males compared with controls, but only at levels of statistical significance in the 500 mg/kg bw per day dose group. Dose-dependent reductions were observed in haematocrit and haemoglobin levels in both sexes of treated animals, with statistical significance attained in males at the three highest dose levels and in females at the two highest dose levels compared with controls. Compared with controls, dose-dependent and statistically significant reductions were also observed in mean corpuscular volumes and levels of mean corpuscular haemoglobin in males at the two highest dose levels and females at the three highest dose levels, as well as in the mean corpuscular haemoglobin concentrations in males at the highest dose level and in females at the two highest dose levels. Males in the three highest dose groups and females in the highest dose group were

reported to have significantly higher levels of reticulocytes than controls. Statistically significant clinical chemistry variations included dose-dependent increases in glutamic–pyruvic transaminase and alkaline phosphatase levels in all groups of treated females (not indicated whether plasma or serum samples were assayed). Creatine phosphokinase levels were increased significantly only in females in the 500 mg/kg bw per day dose group. Blood urea nitrogen levels were reported to be significantly decreased in all test groups of female rats compared with controls. Dose-dependent reductions in creatinine and total protein levels, reaching statistical significance in all female test groups and in the two highest male dose groups, were reported compared with controls. Relative to controls, significantly higher albumin levels were observed in males in the 500 and 1000 mg/kg bw per day dose groups. The ratio of albumin to globulin was also reported to be significantly higher in all groups of treated males and in the two highest female dose groups compared with controls. Relative to controls, glucose levels were significantly elevated in the two highest female dose groups, and total cholesterol and total lipid levels were significantly increased in females and males in the 500 and 1000 mg/kg bw per day dose groups. Potassium levels of males in the 1000 and 2000 mg/kg bw per day dose groups and females in the 250 mg/kg bw per day dose group were reported to be significantly reduced compared with controls. Inorganic phosphorus levels in the 1000 mg/kg bw per day females and in the 2000 mg/kg bw per day males and females were significantly higher than those noted in controls.

Increased liver and kidney weights were also reported in *S*-allyl-L-cysteine-treated rats at dose levels greater than 500 mg/kg bw per day; however, the statistical significance of the organ weight variations was not reported. At necropsy, an incidence of liver adhesion and two incidences of liver hypertrophy were observed in males treated at the 2000 mg/kg bw per day dose level. Spleen surfaces were white in one male and one female in the 2000 mg/kg bw per day dose group. One male in each of the 500 and 2000 mg/kg bw per day dose groups and one female in each of the two highest dose groups exhibited pancreatic atrophy. Furthermore, atrophy of the seminal vesicles and thymus were reported in one male and two females, respectively, in the 2000 mg/kg bw per day dose group. The authors noted, however, that the incidences of these effects in both sexes of treated animals were not significantly different from the respective control groups. Based on the results of this study, the authors determined a NOEL of 250 mg/kg bw per day for *S*-allyl-L-cysteine in rats (Kodera et al., 2002).

(iii) Metabolites and structurally related substances

3-(Methylthio)propionic acid. Groups of male Holtzman rats (five per group) were fed a diet containing 2.57% of the salt of 3-(methylthio)propionic acid for 2 weeks. This dietary concentration corresponded to a calculated (United States Food and Drug Administration, 1993) average daily intake of 2570 mg 3-(methylthio)-propionic acid/kg bw. A concurrent control was maintained. A marked depression of growth and food intake was observed in the 3-(methylthio)propionic acid–treated animals compared with the control rats. The spleens of the treated rats were grossly enlarged and darkened in comparison with the control rats. Histopathological examination of the spleen cells of 3-(methylthio)propionic acid–treated rats by light

and transmission electron microscopy revealed sequestration of large numbers of erythrocytes in splenic sinusoids and red pulp. Marrow changes included increased numbers of erythroblastic islets and electron-dense haemosiderin deposits in islet reticulum cells. Transmission electron microscopic examination of peripheral blood erythrocytes revealed great variation in the size and presence of large numbers of misshapen cells. The peripheral blood erythrocytes had membrane defects and remnants of cytoplasmic organelles (Steele et al., 1979). However, it is not unexpected that high levels of *S*-metabolites would readily sequester haemoglobin and iron and be deposited in the spleen. For example, other sulfur compounds have demonstrated such high-dose effects related to the well recognized reactivity of thiols with haemoglobin and reticulocytes (Munday et al., 1990; Munday & Manns, 1994).

Hydrogen sulfide. In an inhalation study, male and female Sprague-Dawley rats (15 per sex per group) were exposed to hydrogen sulfide concentrations of 14, 43 or 110 mg/m^3 in the air for 6 h/day, 5 days/week, for 90 days. In rats, these inhalation exposure levels correspond to oral administration exposure levels of 2.1, 6.5 and 16.8 mg/kg bw per day, respectively (Fassett, 1978). The rats in the control group were exposed to clean air only. Compared with the control group, reduction in body weight gain was noted at the highest dose level. No significant effects on neurological function (posture and gait, facial tone, papillary reflex, palpebral reflex, extensor thrust reflex and crossed-extensor thrust reflex) were noted in rats exposed to hydrogen sulfide. No treatment-related changes were noted in haematological parameters, in the skeletal muscle, bone marrow or bone, in the spleen or lymph nodes, in the kidneys, in the pituitary, adrenal, thyroid or parathyroid glands or in the skin. Based on the results, the authors determined a NOEL of 43 mg/m^3 (6.5 mg/kg bw per day) in rats (Chemical Industry Institute of Technology, 1983).

Formaldehyde. A NOEL of 15 mg/kg bw per day was reported for formaldehyde added to the drinking-water of male and female Wistar rats (70 per sex per group) for 2 years at dose levels of 0, 1.2, 15 or 82 mg/kg bw per day for males and 0, 1.8, 21 or 109 mg/kg bw for females. There were no adverse effects on general health, survival or haematological or clinical chemistry parameters. Body weight and food intake were decreased in the high-dose group. Treatment-related histopathological gastric changes seen in most of the animals of the high-dose group included papillary epithelial hyperplasia frequently accompanied by hyperkeratosis and focal ulceration in the forestomach and focal chronic atrophic gastritis, occasionally accompanied by ulceration and/or glandular hyperplasia in the glandular stomach. A higher incidence and/or degree of renal papillary necrosis occurred in the high-dose rats. The NOEL appeared to be 15 mg/kg bw per day for males and 21 mg/kg bw per day for females (Til et al., 1989).

(c) Genotoxicity

The results of six additional in vitro tests for genotoxicity for three substances are described below and summarized in Table 5. The results of genotoxicity assays for four structurally related substances are also discussed below.

Table 5. Studies of genotoxicity with simple aliphatic and aromatic sulfides and thiols used as flavouring agents

No.	Flavouring agent	End-point	Test system	Concentration	Results	Reference
In vitro						
1681	Allyl thiohexanoate	Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537	5,[a] 15,[a] 50, 150, 500, 1500 or 5000 µg/plate	Negative[b]	King (2002)
1687	3,6-Diethyl-1,2,4,5-tetrathiane	Reverse mutation[c]	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.1, 0.316, 1, 3.16 or 10 µg/plate	Negative[b]	Uhde (2005)
1687	3,6-Diethyl-1,2,4,5-tetrathiane	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.1, 0.316, 1, 3.16 or 10 µg/plate	Negative[b]	Uhde (2005)
1700	Allyl propyl disulfide	Reverse mutation[d]	*S. typhimurium* TA97, TA98, TA100, TA1535 and TA1537	Up to 333 µg/plate	Negative[b]	Zeiger et al. (1988)
1700	Allyl propyl disulfide[e]	Reverse mutation[f]	*S. typhimurium* TA100	Not specified	Negative[b]	Eder et al. (1982)
1700	Allyl propyl disulfide[g]	Reverse mutation[f]	*S. typhimurium* TA100	0.0015–0.15 µl/ml (1.5–150 µg/ml)[h]	Negative[b]	Eder et al. (1980)

[a] Concentration tested in the absence of metabolic activation.
[b] With and without metabolic activation.
[c] Plate incorporation method.
[d] Preincubation method.
[e] Mixture of 32% allyl propyl disulfide, 31% propyl disulfide and 32% allyl disulfide.
[f] Liquid suspension method.
[g] Mixture of 31% allyl propyl disulfide, 37% propyl disulfide and 32% allyl disulfide.
[h] Calculated based on specific gravity of allyl propyl disulfide (0.999–1.005 g/ml).

(i) In vitro

No evidence of mutagenicity was observed when allyl thiohexanoate (No. 1681), 3,6-diethyl-1,2,4,5-tetrathiane (No. 1687) or allyl propyl disulfide (No. 1700) were incubated with *Salmonella typhimurium* strains TA97, TA98, TA100, TA102, TA1535 and/or TA1537 with and without metabolic activation at concentrations of up to 5000 µg/plate (Eder et al., 1980, 1982; Zeiger et al., 1988; King, 2002; Uhde, 2005).

No evidence of mutagenicity was observed when the structurally related substances tetrahydrothiophene, 2-methylpropane-2-thiol[10] and methyl methanethiosulfonate[11] were incubated with *S. typhimurium* strains TA98, TA100, TA1535, TA1537, TA1538 and/or TA2637 with and without metabolic activation at concentrations of up to 10 000 µg/plate (Dorange et al., 1983; Pennwalt Corporation, 1987a; Phillips Petroleum Company, 1990).

Tetrahydrothiophene tested negative in a cytogenetic assay with human lymphocytes, a mutation assay at the HPRT chromosome with Chinese hamster ovary (CHO) cells and an unscheduled deoxyribonucleic acid (DNA) synthesis test with human epithelial cells performed with and without metabolic activation at concentrations of up to 5120 µg/ml (Pennwalt Corporation, 1987b, 1987c, 1987d).

In the absence of an exogenous metabolic activation system, an increase was observed in the induction of forward mutations when L5178Y*tk*(+/–) mouse lymphoma cells were exposed to 2-methylpropane-2-thiol at the two highest tested concentrations (i.e. 202 and 1000 µg/ml); however, in the presence of such a system, 2-methylpropane-2-thiol yielded negative results at concentrations of up to 1000 µg/ml (Phillips Petroleum Company, 1990). Mouse lymphoma assays conducted in the absence of metabolic activation for simple aliphatic and aromatic substances have been shown to be inconsistent with the results of other standardized genotoxicity assays. Moreover, culture conditions of low pH and high osmolality have been shown to produce false-positive results in in vitro genotoxicity assays (Cifone et al., 1987; Galloway et al., 1987; Heck et al., 1989). Therefore, it is not unexpected that other low molecular weight thiols (e.g. ethyl thiol and butyl thiol) have been shown to produce equivocal or positive evidence of mutagenicity in the mouse lymphoma forward mutation assay, while being negative in reverse

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mutation assays (Eder et al., 1980, 1982; Zeiger et al., 1988; King, 2002; Uhde, 2005). Furthermore, dibutyl disulfide[12] yielded negative results in a mouse lymphoma forward mutation assay without metabolic activation; however, the concentrations tested in this trial were not specified (Dooley et al., 1987).

Tetrahydrothiophene and 2-methylpropane-2-thiol were negative in a sister chromatid exchange (SCE) assay with CHO cells at concentrations of up to 125 and 1350 µg/ml, respectively, with and without metabolic activation (Pennwalt Corporation, no date; Phillips Petroleum Company, 1990). Although a statistically significant increase in the number of SCEs was observed at concentrations of 450 and 1350 µg 2-methylpropane-2-thiol/ml, there was a lack of significant increases at lower test concentrations. Additionally, although statistically significant, the increases in SCEs were less than 2-fold greater than in controls. As such, the authors concluded 2-methylpropane-2-thiol to be non-mutagenic (Phillips Petroleum Company, 1990).

Methyl methanethiosulfonate was negative in chromosomal aberration assays conducted in *Saccharomyces cerevisiae* strain D7 or *S. cerevisiae* haploid strain N123 at concentrations of up to 300 µg/ml (Dorange et al., 1983).

(ii) Conclusion for genotoxicity

The testing of these representative materials in vitro in prokaryotic and eukaryotic test systems indicates that this group of simple aliphatic and aromatic sulfides and thiols is not expected to exhibit any mutagenic or genotoxic properties.

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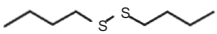
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ALIPHATIC ACYCLIC DIOLS, TRIOLS AND RELATED SUBSTANCES (addendum)

First draft prepared by

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1. EVALUATION

1.1 Introduction

The Committee was requested to evaluate 13 members of a group of aliphatic acyclic diols, triols and related substances. The Committee noted that five substances (listed as Nos 1720, 1721 and 1723–1725) submitted for consideration as members of this group are various fatty acid esters of glycerol and propylene glycol. These substances were previously evaluated by the Committee as emulsifying agents. These substances have specifications and have been allocated acceptable daily intakes (ADIs). Although the use of these substances as flavouring agents would not be anticipated to cause a safety concern, the Committee questioned whether these substances have flavouring properties. The Committee decided not to evaluate them according to the Procedure for the Safety Evaluation

of Flavouring Agents (see Figure 1, Introduction) (Annex 1, reference *131*). In addition, the Committee questioned the flavouring function of lactylated fatty acid esters of glycerol and propylene glycol (listed as No. 1722), for which an ADI and specifications are not available, and decided not to evaluate No. 1722 as a flavouring agent using the Procedure.

From the proposed group, the Committee evaluated seven aliphatic acyclic diols and related substances, including three diol acetals (Nos 1711, 1712 and 1715) and four mono- and dihydroxy derivatives (Nos 1716–1719), using the Procedure.

The Committee previously evaluated 31 other members of this chemical group of flavouring agents at its fifty-seventh meeting (Annex 1, reference *154*). The findings in that report were considered in the present evaluation. The Committee concluded that 17 of the 31 substances in that group were of no safety concern at the estimated current levels of intake. The 17 flavouring agents included two dioxolane derivatives (Nos 838 and 839). The evaluation of the remaining 14 substances could not be finalized, as the Committee requested further data to determine whether these substances are currently used as flavouring agents.

At the subsequent meeting, data on the use of these compounds as flavours were provided to the Committee by the flavour industry (Annex 1, reference *160*). With the exception of glycerol (No. 909) and propylene glycol (No. 925), for which the Committee considered the data provided to be inadequate to substantiate the use of these substances as flavouring agents, the Committee finalized the evaluations of all other agents in this group and concluded that 12 additional flavouring agents were of no safety concern at the estimated current levels of intake as flavouring agents.

At its seventh meeting, the Committee evaluated propylene glycol (No. 925) and assigned an ADI of 0–20 mg/kg body weight (bw) (Annex 1, reference *7*). At the seventeenth meeting, the ADI for propylene glycol (No. 925) was established at 0–25 mg/kg bw (Annex 1, reference *32*). Glycerol, a metabolite of propylene glycol, was evaluated at the twentieth meeting, at which an ADI "not specified" was assigned (Annex 1, reference *41*). In addition to its evaluation for use as a flavouring agent using the Procedure, lactic acid (No. 930), also a metabolite of propylene glycol, was evaluated by the Committee at its seventeenth meeting and allocated an ADI "not limited" (Annex 1, reference *32*).

Three of the seven flavouring agents (Nos 1717–1719) being evaluated at the current meeting have been reported to occur as natural components of food. They have been detected in coffee, mushrooms, pineapple, apple cider and port wine (Nijssen et al., 2006). Consumption ratios (the ratios of their consumption from natural food sources to their use as flavouring agents) were not calculated because no quantitative data were available.

1.2 Assessment of dietary exposure

The total annual volumes of production of the seven aliphatic acyclic diols and related flavouring agents being evaluated at this meeting are approximately 1445 kg in Europe, 53 kg in the United States of America (USA) and 887 kg in Japan

(Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006). Greater than 87% of the annual volume in Europe is accounted for by 2,4-dimethyl-1,3-dioxolane (No. 1711). Greater than 94% of the annual volume in Japan is accounted for by 2,4-dimethyl-1,3-dioxolane (No. 1711), ethyl 2,4-dimethyl-1,3-dioxolane-2-acetate (No. 1715) and dihydroxyacetone dimer (No. 1716). In the USA, dihydroxyacetone dimer (No. 1716) comprises the entire annual volume. The daily per capita intake of each agent is reported in Table 1. Annual volumes of production of this group of flavouring agents are summarized in Table 2.

1.3 Absorption, distribution, metabolism and excretion

Acetals, ketals and esters are hydrolysed to their component alcohols and aldehydes, ketones and acids, respectively. The metabolism of two of the dioxolanes (Nos 1711 and 1715) yields propylene glycol. Propylene glycol is metabolized to endogenous glycerol, lactic acid, pyruvic acid and simple aliphatic alcohols, aldehydes and acids, which are completely metabolized to carbon dioxide and water. In the glycolytic pathway, glycerol is converted to glyceraldehyde-3-phosphate and enters the glycolytic pathway to eventually yield pyruvic acid (Nelson & Cox, 2000). Multiple pathways are available to the other flavouring agents (Nos 1712 and 1716–1719) in this group. These include phosphorylation and conjugation with glucuronic acid (Rudney, 1954).

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure to the above-mentioned seven flavouring agents, the Committee assigned four flavouring agents to structural class I (Nos 1716–1719) and three to structural class III (Nos 1711, 1712 and 1715) (Cramer et al., 1978).

Step 2. All of the flavouring agents in this group are expected to be metabolized to innocuous products. The evaluation of all agents in this group therefore proceeded via the A-side of the Procedure.

Step A3. The estimated daily per capita intakes of all four of the flavouring agents in structural class I are below the threshold of concern (i.e. 1800 µg/person per day for class I). For two flavouring agents (Nos 1712 and 1715) in structural class III, the estimated daily per capita intakes are below the threshold of concern (i.e. 90 µg/person per day for class III). According to the Procedure, the safety of these six flavouring agents raises no concern when they are used at their current estimated levels of intake. For one flavouring agent (No. 1711) in class III, the estimated daily per capita intake exceeds the threshold (i.e. 90 µg/person per day for class III). Accordingly, the evaluation of this flavouring agent proceeded to Step A4.

Table 1. Summary of the results of safety evaluations of aliphatic acyclic diols, triols and related substances used as flavouring agents[a,b,c]

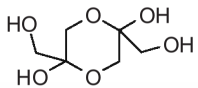
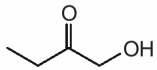
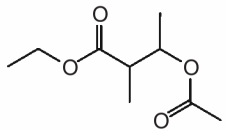
Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class I							
Dihydroxyacetone dimer	1716	62147-49-3 	No Europe: 0.01 USA: 6 Japan: 74	NR	NR	See note 1	No safety concern
1-Hydroxy-2-butanone	1717	5077-67-8 	No Europe: 0.01 USA: ND Japan: 0.08	NR	NR	See note 2	No safety concern
Ethyl 3-acetoxy-2-methylbutyrate	1718	139564-43-5 	No Europe: 0.01 USA: ND Japan: ND	NR	NR	See note 3	No safety concern

Table 1 (Contd)

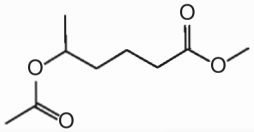
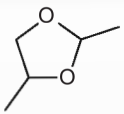
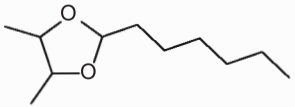
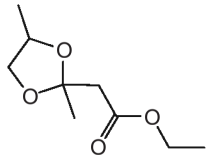
Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Methyl 5-acetoxyhexanoate	1719	35234-22-1 	No Europe: 0.01 USA: ND Japan: ND	NR	NR	See note 3	No safety concern
Structural class III							
2,4-Dimethyl-1,3-dioxolane	1711	3390-12-3 	Yes Europe: 135 USA: ND Japan: 122	No	Yes. The NOEL of 2500 mg/kg bw per day (Gaunt et al., 1972) for the non-endogenous metabolite propylene glycol (No. 925) is >1 million times the estimated intake of 2,4-dimethyl-1,3-dioxolane when used as a flavouring agent.	See note 4	No safety concern
2-Hexyl-4,5-dimethyl-1,3-dioxolane	1712	6454-22-4 	No Europe: 0.01 USA: ND Japan: ND	NR	NR	See note 4	No safety concern

Table 1 (Contd)

Flavouring agent	No.	CAS No. and structure	*Step A3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
cis- and *trans*-Ethyl 2,4-dimethyl-1,3-dioxolane-2-acetate	1715	6290-17-1 	No Europe: 19 USA: ND Japan: 38	NR	NR	See note 5	No safety concern

CAS, Chemical Abstracts Service; ND, no data reported; NR, not required for evaluation because consumption of the substance was determined to be of no safety concern at Step A3 of the Procedure.

[a] Thirty-one flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *155*).

[b] *Step 1:* Four of the flavouring agents in this group are in structural class I, and three are in structural class III.

[c] *Step 2*: All of the flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The threshold for human intakes for structural classes I and III are 1800 and 90 µg/person per day, respectively. All intake values are expressed in µg/day.

Notes:

1. Dihydroxyacetone dimer is readily converted to dihydroxyacetone phosphate, which participates in several metabolic pathways.
2. Detoxicated via conjugation with glucuronic acid and subsequent elimination in the urine.
3. Hydrolysed to the corresponding alcohols and acid, which then enter known pathways of metabolism.
4. Detoxicated by hydrolysis to the corresponding aldehyde and aliphatic glycol, which both are completely metabolized by known pathways.
5. Detoxicated by hydrolysis to the corresponding ketone and aliphatic glycol, which both are completely metabolized by known pathways.

Table 2. Annual volumes of production of aliphatic acyclic diols, triols and related substances used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]	
		µg/day	µg/kg bw per day
2,4-Dimethyl-1,3-dioxolane (1711)			
Europe	1265	135	2
USA	ND	ND	ND
Japan	463	122	2
2-Hexyl-4,5-dimethyl-1,3-dioxolane (1712)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
cis- and *trans*- Ethyl 2,4-dimethyl-1,3-dioxolane-2-acetate (1715)			
Europe	179	19	0.3
USA	ND	ND	ND
Japan	144	38	0.6
Dihydroxyacetone (1716)			
Europe	0.1	0.01	0.0002
USA	53	6	0.1
Japan	279	74	1
1-Hydroxy-2-butanone (1717)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	0.3	0.08	0.001
Ethyl 3-acetoxy-2-methylbutyrate (1718)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
Methyl 5-acetoxyhexanoate (1719)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
Total			
Europe	1445		
USA	53		
Japan	887		

Table 2 (contd)

ND, no data reported.

[a] From European Flavour and Fragrance Association (2005), Flavor and Extract Manufacturers Association (2006) and Japanese Flavor and Fragrance Manufacturers Association (2002). Total poundage values of <1 kg reported in the surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006) have been truncated to one place following the decimal point (0.1 kg).

[b] Intake (μg/person per day) calculated as follows:
[(annual volume, kg) × (1×10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = 32×10^6 for Europe, 28×10^6 for the USA and 13×10^6 for Japan; and where correction factor = 0.8 for the surveys in the USA, Europe and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006).

Step A4. Although one metabolite of No. 1711 is an endogenous substance (acetic acid), the other metabolite (propylene glycol) is not. Accordingly, the evaluation of this flavouring agent proceeded to Step A5.

Step A5. The no-observed-effect level (NOEL) of 2500 mg/kg bw per day for propylene glycol (No. 925) from a 2-year study of toxicity in rats (Gaunt et al., 1972) is >1 million times the estimated intake of the parent compound 2,4-dimethyl-1,3-dioxolane (No. 1711) from its use as a flavouring agent. The Committee therefore concluded that 2,4-dimethyl-1,3-dioxolane would not pose a safety concern at the currently estimated level of intake.

Table 1 summarizes the stepwise evaluation of these seven flavouring agents.

1.5 Consideration of combined intakes from use as flavouring agents

The flavouring agents in this group are predicted to be metabolized by hydrolysis, pathways of intermediate metabolism and/or conjugation. These pathways have a high capacity and would not be saturated, even if all flavouring agents were consumed at the same time. A number of the flavouring agents in this group that have been evaluated at this meeting and at the fifty-seventh meeting are predicted to be hydrolysed to common intermediary metabolites, such as lactate and pyruvate. The Committee concluded that combined intakes would not raise safety concerns.

1.6 Consideration of secondary components

One flavouring agent in this group, 1-hydroxy-2-butanone (No. 1717), has a minimum assay value of <95%. Information on the safety of the secondary component of this compound is summarized in Annex 5 (Summary of the safety

evaluation of secondary components for flavouring agents with minimum assay values of less than 95%). The secondary component of 1-hydroxy-2-butanone is acetoin (No. 405), which is expected to share the same metabolic fate as 1-hydroxy-2-butanone. Acetoin (No. 405) was evaluated by the Committee for its use as a flavouring agent at the fifty-first meeting (Annex 1, reference *137*) and considered not to present a safety concern at current levels of intake.

1.7 Conclusion

In the previous evaluation of flavouring agents in this group of aliphatic acyclic diols, triols and related substances, studies of acute toxicity, short-term toxicity, long-term toxicity and carcinogenicity and genotoxicity were available. None raised safety concerns. The toxicity data available for this evaluation were supported by those from previous evaluations at the fifty-seventh meeting.

The Committee concluded that these seven flavouring agents, which are additions to the group evaluated previously, would not give rise to safety concerns at the currently estimated levels of intake. Six other substances proposed for evaluation in this group were not evaluated using the Procedure, as the Committee questioned whether they had flavouring properties.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of seven flavouring agents that are additions to the group of aliphatic acyclic diols, triols and related substances evaluated previously (see Table 1). Six other substances proposed for evaluation in this group were not evaluated, as the Committee questioned whether they had flavouring properties.

2.2 Additional considerations on intake

There is no additional information on intake.

2.3 Biological data

2.3.1 Biochemical data: absorption, distribution, metabolism and excretion

(a) Dihydroxy derivatives

Dihydroxyacetone (No. 1716) is classified chemically as a ketotriose. It is endogenous in animals and is readily phosphorylated in vivo to form dihydroxyacetone phosphate. Dihydroxyacetone phosphate is involved in several common metabolic pathways, glycolysis and gluconeogenesis, and the glycerophosphate shuttle (Michal, 1999). It is also part of the Calvin cycle in plant metabolism (Michal, 1999).

Diets intended to induce weight gain of nine female volunteers were supplemented with 75 g dihydroxyacetone (No. 1716)/day together with 15 g pyruvate provided in water or a placebo for 21 days. Prior to the supplementation, subjects had been maintained on hypoenergetic diets. All urine and stools were collected and analysed for nitrogen and protein content. Blood samples were drawn on days 1 and 21. Complete haematological analysis was performed. At day 21, blood glucose, insulin, cholesterol and triglyceride levels had increased significantly compared with the baseline levels in both the placebo and dihydroxyacetone-treated group; however, there were no differences in the parameters between the two groups (Stanko & Arch, 1996).

2.2.2 Toxicological studies

(a) Acute toxicity

An oral LD_{50} value has been reported for one (No. 1716) of the seven substances in this group, as summarized in Table 3. In rats, the LD_{50} value for dihydroxyacetone (No. 1716) was >60 000 mg/kg bw (Laborit, 1977), demonstrating that the oral acute toxicity in this group of flavouring agents is extremely low.

Table 3. Results of oral acute toxicity studies with aliphatic acyclic diols, triols and related substances used as flavouring agents

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1716	Dihydroxyacetone	Rat; NR	>60 000	Laborit (1977)

NR, not reported.

(b) Short-term studies of toxicity

Short-term toxicity studies have been reported for one substance (No. 1716) in this group, as summarized in Table 4.

Table 4. Results of short-term studies of toxicity with aliphatic acyclic diols, triols and related substances used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1716	Dihydroxyacetone	Rat; F	1/6	Diet	35	6000[b]	Cortez et al. (1991)

F, female.

[a] Total number of test groups does not include control animals.

[b] Study performed with either a single dose or multiple doses that had produced no adverse effect. The value is therefore not a true NOEL, but is the highest dose level tested that produced no adverse effects. The actual NOEL may be higher.

(i) Dihydroxyacetone (No. 1716)

In a 35-day study, a group of female obese Zucker rats ($n = 6$) was fed a diet delivering 6% dihydroxyacetone (6% group). This dietary level corresponds to an estimated daily intake of 6000 mg dihydroxyacetone/kg bw in rats. An additional group of female obese Zucker rats ($n = 6$) was administered a mixture providing 3% each of pyruvate and dihydroxyacetone (3% group). The flavouring agents were substituted for part of the dextrose in the diets. Two control groups of female obese Zucker rats ($n = 6$ per group) were included in this study, with one control group provided the basal diet ad libitum and the other pair-fed to the lowest level of intake observed in the test groups. Blood was drawn for analysis during the 3rd week and at the termination of the study. At study end, animals were killed, and a portion of the left liver lobe was removed for analysis of fat and glycogen content. The remainder of the liver as well as the heart, retroperitoneal fat pads and left leg muscles were also removed and weighed. Final body weights and body weight gain of the 6% and 3% groups were significantly decreased compared with the ad libitum control group. The 6% and 3% groups of rats exhibited significantly decreased food conversion efficiencies compared with the ad libitum control animals, as well as the pair-fed controls in the case of the 3% group. Total plasma cholesterol levels were increased in the 3% treated rats compared with both controls. Plasma high-density lipoprotein cholesterol in both test groups and plasma triglyceride levels in the 3% group were increased and decreased, respectively, compared with the ad libitum control group. Absolute (mmol) and relative (µmol/g wet weight) liver triglyceride levels were increased in both treatment groups compared with the ad libitum control animals, whereas relative liver glycogen levels were significantly reduced in the 3% group rats. Absolute liver triglyceride levels were significantly elevated in the 6% group rats compared with the pair-fed controls. The absolute weights of the gastrocnemius and plantaris leg muscles of both treated groups were significantly decreased compared with the ad libitum controls and pair-fed controls for the plantaris muscle. The gastrocnemius muscle weight was significantly decreased compared with the pair-fed controls only in the 3% group. In the 3% treatment group of rats, absolute heart weight was decreased compared with the ad libitum controls, and absolute liver weight was increased compared with both sets of controls. In both groups of treated rats, relative liver weights were increased significantly compared with controls; however, while the increase in the 3% group of rats was significant compared with both controls, the increase in the 6% group of rats was significant only in comparison with the ad libitum control group. In summary, the most notable effect of dihydroxyacetone administration to obese Zucker rats was steatosis of the liver, which was not present in either of the control groups (Cortez et al., 1991).

(c) Genotoxicity

A genotoxicity study has been reported for No. 1716 in this group and is summarized in Table 5.

Table 5. In vitro studies of genotoxicity with aliphatic acyclic diols, triols and related substances used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
1716	Dihydroxy acetone	Reverse mutation	*Salmonella typhimurium* TA102 and TA104	1000 µg/plate	Negative[a]	Marnett et al. (1985)

[a] With and without metabolic activation.

In a modified reverse mutation assay using the pre-incubation method, no mutagenic activity was observed when *Salmonella typhimurium* strains TA102 and TA104 were incubated in the presence of 1000 µg dihydroxyacetone (No. 1716)/plate with and without metabolic activation (Marnett et al., 1985).

In vitro testing of these representative materials in bacterial and mammalian test systems showed no evidence of mutagenic or genotoxic potential.

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SULFUR-CONTAINING HETEROCYCLIC COMPOUNDS (addendum)

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 17 flavouring agents comprising sulfur-containing heterocyclic compounds using the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, Introduction) (Annex 1, reference *131*). The group was composed of both five- and six-member sulfur-containing aromatic and non-aromatic heterocyclic compounds, including three thiophene (Nos 1750, 1764 and 1765), eight thiazole (Nos 1751, 1752, 1753, 1754, 1755, 1756, 1757 and 1758), four thiazoline (Nos 1759, 1760, 1761 and 1762), one thiazine (No. 1766) and one dithiazine (No. 1763) derivative. The Committee has not evaluated these flavouring agents previously.

The Committee previously evaluated 30 other members of this chemical group of flavouring agents at its fifty-ninth meeting (Annex 1, reference *160*). The

findings presented in that report were considered in the present evaluation. For all 30 substances, the Committee concluded that there were no safety concerns at the currently estimated levels of intake.

Three of the 17 flavouring agents (Nos 1758, 1759 and 1764) in this group have been reported to occur naturally in coffee, black tea, barley, chicken, turkey, guinea hen, beef, mushrooms, trassi, American cranberry and sweet corn (Nijssen et al., 2006). No quantitative data on the natural levels in food were available, and therefore consumption ratios (the ratios of their consumption from natural food sources to their use as flavouring agents) were not calculated.

1.2 Assessment of dietary exposure

The total annual volume of production of this group of sulfur-containing heterocyclic compounds is approximately 86 kg in Europe, 2 kg in the United States of America (USA) and 1145 kg in Japan (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006). Approximately 88% of the total annual volume of production in Europe is accounted for by 2-acetyl-2-thiazoline (No. 1759). The daily per capita intake of 2-acetyl-2-thiazoline (No. 1759) is 8 µg/kg in Europe and 4 µg/kg in Japan. Approximately 94% of the total annual volume of production in Japan is accounted for by four flavouring agents: 2-(4-methyl-5-thiazolyl)ethyl butanoate (No. 1753), 2-(4-methyl-5-thiazolyl)ethyl isobutyrate (No. 1754), 2-(4-methyl-5-thiazolyl)ethyl octanoate (No. 1756) and 2-(4-methyl-5-thiazolyl)ethyl decanoate (No. 1757). The daily per capita intake of each flavouring agent is reported in Table 1. Annual volumes of production of this group of flavouring agents are reported in Table 2.

1.3 Absorption, distribution, metabolism and elimination

The metabolism of sulfur-containing heterocyclic compounds was previously described in the report of the fifty-ninth meeting. Thiazole and its derivatives are metabolized primarily by side-chain oxidation or oxidation of the ring sulfur or nitrogen atoms (Rance, 1989); however, other routes of metabolism, involving ring cleavage, are possible. Seven of the thiazole derivatives in this group are 2-thiazolylethyl esters (Nos 1751–1757) and are hydrolysed to form 4-methyl-5-thiazoleethanol (No. 1031), a normal metabolite of vitamin B_1 (thiamine), which is further oxidized and excreted free or as a glutathione conjugate (Tietz, 1986).

Thiazoline derivatives (Nos 1759–1762), being cyclic sulfides, are metabolized primarily by *S*-oxidation to yield corresponding sulfoxides and sulfones (Nelson & Cox, 2000).

Thiophene derivatives are subject to *S*-oxidation followed by conjugation with glutathione; however, other routes of metabolism, involving ring cleavage, are also possible. The resulting mercapturic acid derivative is eliminated in the urine (Dansette et al., 1992; Valadon et al., 1996).

Thiazine and dithiazine derivatives are expected to be metabolized primarily via side-chain oxidation and ring *S*- and *N*-oxidation.

Table 1. Summary of the results of safety evaluations of sulfur-containing heterocyclic compounds used as flavouring agents[a,b,c]

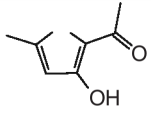
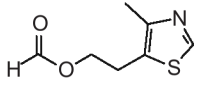
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class II								
1-(3-Hydroxy-5-methyl-2-thienyl)ethanone	1750	133860-42-1 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 290 mg/kg bw per day (Morgareidge & Oser, 1970) for the related substance 2-thienyldisulfide (No. 1053) is >1 billion times the estimated intake of 1-(3-hydroxy-5-methyl-2-thienyl)ethanone when used as a flavouring agent.	See note 3	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl formate	1751	90731-56-9 	Yes—A-side	No Europe: ND USA: ND Japan: 2	NR	NR	See note 2	No safety concern

Table 1 (contd)

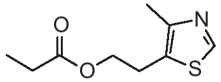
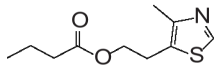
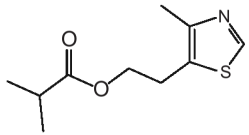
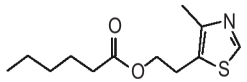
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
2-(4-Methyl-5-thiazolyl)ethyl propionate	1752	324742-96-3 	Yes—A-side	No Europe: ND USA: ND Japan: 10	NR	NR	See note 2	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl butanoate	1753	94159-31-6 	Yes—A-side	No Europe: ND USA: ND Japan: 78	NR	NR	See note 2	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl isobutyrate	1754	324742-95-2 	Yes—A-side	No Europe: ND USA: ND Japan: 31	NR	NR	See note 2	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl hexanoate	1755	94159-32-7 	Yes—A-side	No Europe: ND USA: ND Japan: 4	NR	NR	See note 2	No safety concern

Table 1 (contd)

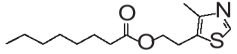
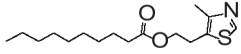
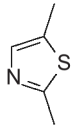
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
2-(4-Methyl-5-thiazolyl)ethyl octanoate	1756	163266-17-9 	Yes—A-side	No Europe: ND USA: ND Japan: 144	NR	NR	See note 2	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl decanoate	1757	101426-31-7 	Yes—A-side	No Europe: ND USA: ND Japan: 30	NR	NR	See note 2	No safety concern
2,5-Dimethylthiazole	1758	4175-66-0 	No—B-side	No Europe: 0.01 USA: ND Japan: 0.03	NR	Yes. The NOEL of 0.93 mg/kg bw per day (Posternak et al., 1969) for the related substance 2,4-dimethyl-5-vinylthiazole (No. 1039) is >2 million times the estimated intake of 2,5-dimethylthiazole when used as a flavouring agent.	See note 1	No safety concern

Table 1 (contd)

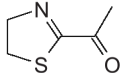
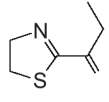
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
2-Acetyl-2-thiazoline	1759	29926-41-8 	No—B-side	No Europe: 8 USA: ND Japan: 4	NR	Yes. The NOEL of 1.8 mg/kg bw per day (Munday & Kirkby, 1971b) is 18 000 and 30 000 times the estimated daily intake of 2-acetyl-2-thiazoline when used as a flavouring agent in Europe and Japan, respectively.	See note 1	No safety concern
2-Propionyl-2-thiazoline	1760	29926-42-9 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 1.2 mg/kg bw per day (Babish, 1978) for the related substance 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) is 6 million times the estimated intake of 2-propionyl-2-thiazoline when used as a flavouring agent.	See note 1	No safety concern

Table 1 (contd)

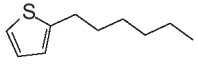
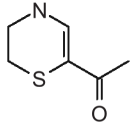
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
2-Hexylthiophene	1764	18794-77-9 	No—B-side	No Europe: 1 USA: 0.1 Japan: ND	NR	Yes. The NOEL of 290 mg/kg bw per day (Morgareidge & Oser, 1970) for the related substance 2-thienyldisulfide (No. 1053) is >14 million times the estimated intake of 2-hexylthiophene when used as a flavouring agent.	See note 3	No safety concern
5-Acetyl-2,3-dihydro-1,4-thiazine	1766	164524-93-0 	No—B-side	No Europe: ND USA: 0.2 Japan: ND	NR	Yes. The NOEL of 11 mg/kg bw per day (Rush, 1989a, 1989b) for the mixture of related substances 2-isobutyl-4,6-dimethyldihydro-1,3,5-dithiazine and 4-isobutyl-2,6-	See note 1	No safety concern

Table 1 (contd)

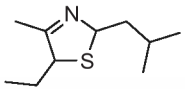
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
						dimethyldihydro-1,3,5-dithiazine (No. 1046) is >3 million times the estimated intake of 5-acetyl-2,3-dihydro-1,4-thiazine when used as a flavouring agent.		
Structural class III								
cis- and *trans*-5-Ethyl-4-methyl-2-(2-methylpropyl)-thiazoline	1761	83418-53-5 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 1.2 mg/kg bw per day (Babish, 1978) for the related substance 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) is 6 million times the estimated intake of *cis*- and *trans*-5-ethyl-4-methyl-2-(2-methylpropyl)-thiazoline when used as a flavouring agent.	See note 1	No safety concern

Table 1 (contd)

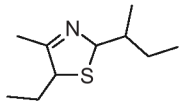
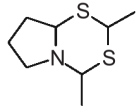
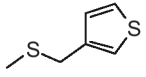
Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
cis- and *trans*-5-Ethyl-4-methyl-2-(1-methylpropyl)-thiazoline	1762	83418-54-6 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 1.2 mg/kg bw per day (Babish, 1978) for the related substance 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) is 6 million times the estimated intake of *cis*- and *trans*-5-ethyl-4-methyl-2-(2-butyl)-thiazoline when used as a flavouring agent.	See note 1	No safety concern
Pyrrolidino-[1,2*e*]-4H-2,4-dimethyl-1,3,5-dithiazine	1763	116505-60-3 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 11 mg/kg bw per day (Rush, 1989a, 1989b) for the mixture of related substances 2-isobutyl-4,6-dimethyldihydro-1,3,5-dithiazine and 4-isobutyl-2,6-dimethyldihydro-1,3,5-dithiazine (No. 1046) is	See note 1	No safety concern

Table 1 (contd)

Flavouring agent	No.	CAS No. and structure	*Step 2* Predicted to be metabolized to innocuous products?	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5/B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
						55 million times the estimated intake of pyrrolidino-[1,2*e*]-4H-2,4-dimethyl-1,3,5-dithiazine when used as a flavouring agent.		
3-(Methylthio)-methylthiophene	1765	61675-72-7 	No—B-side	No Europe: 0.01 USA: ND Japan: ND	NR	Yes. The NOEL of 290 mg/kg bw per day (Morgareidge & Oser, 1970) for the related substance 2-thienyldisulfide (No. 1053) is >1 billion times the estimated intake of 3-(methylthio)-methylthiophene when used as a flavouring agent.	See note 3	No safety concern

CAS, Chemical Abstracts Service; ND, no data reported; NR, not required for evaluation because consumption of the substance was determined to be of no safety concern at Step A3 of the Procedure.

Table 1 (contd)

[a] Thirty flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *160*).
[b] *Step 1*: Ten flavouring agents are in structural class II, and seven are in structural class III.
[c] *Step 2:* All of the agents in this group cannot be predicted to be metabolized to innocuous products.
[d] The thresholds for human intake for structural classes II and III are 540 and 90 µg/day, respectively. All intake values are expressed in µg/day.

Notes:
1. Metabolized primarily by side-chain oxidation and/or ring *S*- or *N*-oxidation. The major metabolites are readily excreted in the urine either free or as glucuronide or sulfate conjugates.
2. Enzymatically cleaved to yield 4-methyl-5-thiazoleethanol (No. 1031) and 2-methyl-4-amino-5-hydroxymethylpyrimidine. The thiazole and pyrimidine fragments are further oxidized to yield 4-methylthiazole-4-acetic acid and the 5-pyrimidine carboxylic acid derivative, respectively, which, together with thiamine, are excreted in the urine. May also be converted to 2-methyl-4-amino-5-formylaminopyrimidine and thiamine acetic acid.
3. Metabolized primarily by side-chain and/or ring *S*-oxidation followed by glutathione conjugation and elimination in the urine.

Table 2. Annual volumes of production of sulfur-containing heterocyclic compounds used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]	
		µg/day	µg/kg bw per day
1-(3-Hydroxy-5-methyl-2-thienyl)ethanone (1750)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
2-(4-Methyl-5-thiazolyl)ethyl formate (1751)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	7	2	0.03
2-(4-Methyl-5-thiazolyl)ethyl propionate (1752)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	37	10	0.2
2-(4-Methyl-5-thiazolyl)ethyl butanoate (1753)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	297	78	1
2-(4-Methyl-5-thiazolyl)ethyl isobutyrate (1754)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	119	31	0.5
2-(4-Methyl-5-thiazolyl)ethyl hexanoate (1755)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	14	4	0.07
2-(4-Methyl-5-thiazolyl)ethyl octanoate (1756)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	545	144	2

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]	
		µg/day	µg/kg bw per day
2-(4-Methyl-5-thiazolyl)ethyl decanoate (1757)			
Europe	ND	ND	ND
USA	ND	ND	ND
Japan	113	30	0.5
2,5-Dimethylthiazole (1758)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	0.1	0.03	0.0005
2-Acetyl-2-thiazoline (1759)			
Europe	76	8	0.1
USA	ND	ND	ND
Japan	13	4	0.07
2-Propionyl-2-thiazoline (1760)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
cis- and *trans*-5-Ethyl-4-methyl-2-(2-methylpropyl)-thiazoline (1761)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
cis- and *trans*-5-Ethyl-4-methyl-2-(1-methylpropyl)-thiazoline (1762)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
Pyrrolidino-[1,2e]-4H-2,4-dimethyl-1,3,5-dithiazine (1763)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
2-Hexylthiophene (1764)			
Europe	10	1	0.02
USA	1	0.1	0.002
Japan	ND	ND	ND

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]	
		µg/day	µg/kg bw per day
3-(Methylthio)methylthiophene (1765)			
Europe	0.1	0.01	0.0002
USA	ND	ND	ND
Japan	ND	ND	ND
5-Acetyl-2,3-dihydro-1,4-thiazine (1766)			
Europe	ND	ND	ND
USA	1	0.2	0.003
Japan	ND	ND	ND
Total			
Europe	86		
USA	2		
Japan	1145		

ND, no data reported.

[a] From European Flavour and Fragrance Association (2005), Flavor and Extract Manufacturers Association (2006) and Japanese Flavor and Fragrance Manufacturers Association (2002). Total poundage values of <1 kg reported in the surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006) have been truncated to one place following the decimal point (0.1 kg).

[b] Intake (µg/person per day) calculated as follows:
[(annual volume, kg) × (1×10^9 µg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = 32×10^6 for Europe, 28×10^6 for the USA and 13×10^6 for Japan; and where correction factor = 0.8 for surveys in Europe, the USA and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006).
Intake (µg/kg bw per day) calculated as follows:
(µg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur as a result of rounding.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned 13 flavouring agents (Nos 1750–1760, 1764 and 1766) to structural class II and 4 flavouring agents (Nos 1761–1763 and 1765) to structural class III (Cramer et al., 1978).

Step 2. The seven 2-thiazolylethyl esters (Nos 1751–1757) are expected to be readily hydrolysed to the thiamine metabolite, 2-(4-methyl-5-thiazolyl)ethanol (No. 1031), which in turn is metabolized to an innocuous product. The evaluation of the seven esters therefore proceeded via the A-side of the Procedure. For the remaining 10 flavouring agents in this group (Nos 1750 and 1758–1766), the data were insufficient to allow conclusions about their probable metabolic fate. Therefore, for these flavouring agents, the evaluation proceeded via the B-side of the Procedure.

Step A3. With regard to those seven flavouring agents (Nos 1751–1757) evaluated via the A-side of the Procedure, the estimated daily per capita intakes are below the threshold of concern for the structural class (i.e. 540 µg/person per day for class II). According to the Procedure, there are no safety concerns for these seven flavouring agents when used at the currently estimated levels of intake.

Step B3. With regard to those 10 flavouring agents evaluated via the B-side of the Procedure, the estimated daily per capita intakes of the 6 flavouring agents in structural class II (Nos 1750, 1758–1760, 1764 and 1766) are below the threshold of concern (i.e. 540 µg/person per day for class II). Similarly, the estimated daily per capita intakes for the 4 flavouring agents in structural class III (Nos 1761–1763 and 1765) are below the threshold of concern (i.e. 90 µg/person per day for class III). The evaluation of these 10 flavouring agents therefore proceeded to step B4.

Step B4. The no-observed-effect level (NOEL) for 2-thienyldisulfide (No. 1053) in a 90-day dietary study in rats was 290 mg/kg bw per day (Morgareidge & Oser, 1970), and this NOEL is appropriate to evaluate the structurally related flavouring agents 1-(3-hydroxy-5-methyl-2-thienyl)ethanone (No. 1750), 3-(methylthio)methylthiophene (No. 1765) and 2-hexylthiophene (No. 1764). The NOEL is >1 billion times the estimated intake of 1-(3-hydroxy-5-methyl-2-thienyl)ethanone and 3-(methylthio)methylthiophene from their use as flavouring agents in Europe (0.0002 µg/kg bw per day). The NOEL is >14 million times the estimated intake of 2-hexylthiophene from its use as a flavouring agent in Europe (0.02 µg/kg bw per day).

The NOEL for 2,4-dimethyl-5-vinylthiazole (No. 1039) in a 90-day study in rats was 0.93 mg/kg bw per day (Posternak et al., 1969), and this NOEL is appropriate to evaluate the structurally related flavouring agent 2,5-dimethylthiazole (No. 1758). The NOEL is >2 million times the estimated intake of 2,5-dimethylthiazole from its use as a flavouring agent in Japan (0.0004 µg/kg bw per day).

The NOEL for 2-isobutyl-4,6-dimethyldihydro-1,3,5-dithiazine and 4-isobutyl-2,6-dimethyldihydro-1,3,5-dithiazine (mixture) (No. 1046) in a 90-day study in rats was 11 mg/kg bw per day (Rush, 1989a, 1989b),

and this NOEL is appropriate to evaluate the structurally related flavouring agents pyrrolidino-[1,2e]-4H-2,4-dimethyl-1,3,5-dithiazine (No. 1763) and 5-acetyl-2,3-dihydro-1,4-thiazine (No. 1766). The NOEL is 55 million times the estimated intake of pyrrolidino-[1,2e]-4H-2,4-dimethyl-1,3,5-dithiazine from its use as a flavouring agent in Europe (0.0002 µg/kg bw per day). The NOEL is >3 million times the estimated intake of 5-acetyl-2,3-dihydro-1,4-thiazine from its use as a flavouring agent in the USA (0.003 µg/kg bw per day).

For 2-acetyl-2-thiazoline (No. 1759), the NOEL of 1.8 mg/kg bw per day from a 90-day rat study that examined the toxicity of a cocktail of flavours (Munday & Kirkby, 1971b) provides a margin of safety of 18 000 and 30 000 in relation to the estimated levels of exposure from its use as a flavouring agent in Europe (0.1 µg/kg bw per day) and in Japan (0.06 µg/kg bw per day).

The NOEL for 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) in a 90-day study in male rats was 1.2 mg/kg bw per day (Babish, 1978), and this NOEL is appropriate to evaluate the structurally related flavouring agents 2-acetyl-2-thiazoline (No. 1759), 2-propionyl-2-thiazoline (No. 1760), *cis*- and *trans*-5-ethyl-4-methyl-2-(2-methylpropyl)thiazoline (No. 1761) and *cis*- and *trans*-5-ethyl-4-methyl-2-(1-methylpropyl)thiazoline (No. 1762). The NOEL is 12 000 times the estimated intake of 2-acetyl-2-thiazoline from its use as a flavouring agent in Europe (0.1 µg/kg bw per day). The NOEL is 6 million times the estimated intakes of 2-propionyl-2-thiazoline, *cis*- and *trans*-5-ethyl-4-methyl-2-(2-methylpropyl)thiazoline and *cis*- and *trans*-5-ethyl-4-methyl-2-(1-methylpropyl)thiazoline from their use as flavouring agents in Europe (0.0002 µg/kg bw per day).

The NOEL for 2-acetyl-2-thiazole (No. 1041) in a 28-day study in rats was 50 mg/kg bw per day (Annex 1, reference *160*), and this NOEL is also appropriate to evaluate the structurally related flavouring agents 2-acetyl-2-thiazoline (No. 1759) and 2-propionyl-2-thiazoline (No. 1760). The NOEL is 500 000 times the estimated intake of 2-acetyl-2-thiazoline and 250 million times the estimated intake of 2-propionyl-2-thiazoline from their use as flavouring agents.

Table 1 summarizes the evaluations of the 17 sulfur-containing heterocyclic flavouring agents in this group.

1.5 Consideration of combined intakes from use as flavouring agents

The flavouring agents in this group are predicted to be metabolized by hydrolysis, by oxidative metabolism of heterocyclic rings and/or alkyl side-chains and by conjugation with glucuronic acid and/or glutathione. These pathways have a high capacity and would not be saturated, even if all flavouring agents were consumed at the same time. A number of the substances in this group that have been evaluated at this meeting and at the fifty-ninth meeting are predicted to be metabolized to a common metabolite. 4-Methyl-5-thiazoleethanol (No. 1031) is a

predicted metabolite of Nos 1751–1757, and the combined intake would be below the threshold for class II. The other substances in this group have diverse structures, with various potential sites of metabolism, and are not likely to be metabolized to common products. The Committee concluded that under the conditions of use as flavouring agents, the combined intake of these substances would not saturate the metabolic pathways and the combined intakes would not raise safety concerns.

1.6 Consideration of secondary components

No flavouring agents in this group have minimum assay values of <95%.

1.7 Conclusion

In the previous evaluation of substances in this group, studies of acute toxicity, short-term toxicity and genotoxicity were available. None raised safety concerns. The toxicity data available for this evaluation were supported by those from the previous evaluation.

The Committee concluded that these 17 flavouring agents, which are additions to the group of sulfur-containing heterocyclic compounds evaluated previously, would not give rise to safety concerns at the currently estimated levels of intake.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes the key data relevant to the safety evaluation of 17 flavouring agents that are additions to the group of sulfur-containing heterocyclic compounds evaluated previously (see Table 1). The group was composed of both five- and six-member sulfur-containing aromatic and non-aromatic heterocyclic compounds, including three thiophene, eight thiazole, four thiazoline, one thiazine and one dithiazine derivative.

2.2 Additional considerations on intake

There is no additional information on the estimated daily intake of this group of flavouring agents.

2.3 Biological data

2.3.1 Biochemical data: absorption, distribution, metabolism and excretion

There has been no additional information on the absorption, distribution, metabolism or excretion of this group of flavouring agents since the report of the fifth-ninth meeting (Annex 1, reference *160*).

2.3.2 Toxicological studies

(a) Acute toxicity

There has been no additional information on the acute toxicity of this group of flavouring agents since the report of the fifty-ninth meeting (Annex 1, reference 160).

(b) Short-term studies of toxicity

Short-term studies of toxicity on one substance in this group are summarized in Table 3 and discussed below. Also discussed is a short-term study on toxicity for a structurally related substance.

(i) 2-Acetyl-2-thiazoline (No. 1759)

Three-week, 6-week, 13-week and 1-year studies in rats were conducted.

In a palatability trial, groups of three male and three female rats (strain not specified) were fed a control diet for 1 week followed by diets containing 197, 492,

Table 3. Results of short-term studies of toxicity with sulfur-containing heterocyclic compounds used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1759	2-Acetyl-2-thiazoline[c]	Rat; M, F	6/6	Diet	21	1.8	Munday &Kirkby (1971a)
1759	2-Acetyl-2-thiazoline[c]	Rat; M, F	3/12	Diet	42	1.8	Munday &Kirkby (1971b)
1759	2-Acetyl-2-thiazoline[c]	Rat; M, F	6/16	Diet	90	1.8	Munday &Kirkby (1971b)
1759	2-Acetyl-2-thiazoline[c,d]	Rat; M, F	1/96	Diet	350	1.8	Munday &Kirkby (1973)

F, female; M, male.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] Study performed using a flavour cocktail of 74.1% 4-hydroxy-5-methyl-3(2H)-furanone (No. 1450), 23.2% 2,3-dimethyl-4-hydroxy-2,5-dihydrofuran-5-one, 1.8% 2-acetyl-2-thiazole (No. 1041) and 0.9% 2-acetyl-2-thiazoline (No. 1759) at a concentration of up to 3932 mg/kg diet (a dose of approximately 197 mg/kg bw of the flavour cocktail).

[d] Rats were maintained on diets containing a flavour cocktail equivalent to approximately 0.09, 0.2, 0.4, 0.9, 1.3 or 1.8 mg 2-acetyl-2-thiazoline (No. 1759)/kg bw per day for the first 13 weeks, and subsequently only on a diet at the highest level of 1.8 mg 2-acetyl-2-thiazoline/kg bw per day for an additional 37 weeks.

983, 1966, 2949 or 3932 mg/kg diet of a flavour cocktail, which included 74.1% 4-hydroxy-5-methyl-3(2H)-furanone (No. 1450), 23.2% 2,3-dimethyl-4-hydroxy-2,5-dihydrofuran-5-one, 1.8% 2-acetyl-2-thiazole (No. 1041) and 0.9% 2-acetyl-2-thiazoline, for a period of 3 weeks. These dietary levels provided average daily intakes of approximately 10, 25, 49, 98, 147 and 197 mg/kg bw of the flavour cocktail, which were equivalent to approximately 0.09, 0.2, 0.4, 0.9, 1.3 and 1.8 mg 2-acetyl-2-thiazoline/kg bw per day, respectively. A group of six male and six female rats was fed a control diet throughout the 4-week study period. Based on measurements of body weight, food and water consumption and food utilization taken 2 or 3 times weekly, no consistent significant differences were observed between test and control animals over the 4-week study period. Haematological examination (haematocrit and total white blood cell counts) of all rats at the end of the study revealed a significant increase (2–4%) in haematocrit in male rats fed 492, 983, 1966 or 2949 mg/kg and in female rats fed 492 or 983 mg/kg of the flavour cocktail. Total white blood cell counts of female rats administered 983 mg/kg of the flavour cocktail were also significantly greater compared with controls. There was no dose–response relationship in these effects, and they were regarded as not biologically significant. Upon necropsy at study termination, the absolute and relative organ weights (i.e. liver, spleen, heart, kidneys and testes) of male rats were not significantly different between test and control groups. High-dose female rats (3932 mg/kg of the flavour cocktail) exhibited a small but significant increase in relative liver weights compared with controls, and females in the 492 mg/kg group showed a small but significant decrease in relative spleen and kidney weights; however, macroscopic examination of the tissues of all animals at postmortem showed no significant findings attributable to the flavour cocktail (Munday & Kirkby, 1971a).

In a follow-up study, groups of eight male and eight female weanling Colworth Wistar rats were maintained on diets containing 197, 492, 983, 1966, 2949 or 3932 mg/kg of the same flavour cocktail used in the previous study (Munday & Kirkby, 1971a) for a period of 13 weeks. A control group of 16 males and 16 females was maintained on a basal diet for 13 weeks. These dietary levels provided average daily intakes of approximately 10, 25, 49, 98, 147 and 197 mg/kg bw of the flavour cocktail, which were equivalent to approximately 0.09, 0.2, 0.4, 0.9, 1.3 and 1.8 mg 2-acetyl-2-thiazoline/kg bw per day, respectively. Biochemical tests were performed at 13 weeks on both sexes of rats maintained on the 492, 983 and 2949 mg/kg diets. Haematological examination was performed at 6 and 13 weeks on groups of animals maintained on the 197, 1966 and 3932 mg/kg diets. All animals were subjected to postmortem examination, organ weight measurements and histological examination of organs.

In addition, separate groups of male and female rats (six per sex per group) were fed diets containing 197, 1966 and 3932 mg/kg of the flavour cocktail for a period of 6 weeks. A control group of 12 male and 12 female rats was maintained on a basal diet for 6 weeks. At the end of the 6-week period, all animals were killed, and organ weight measurements and macroscopic and microscopic examinations were performed. Biochemical tests on blood and urine were conducted on this

group of animals, as well as organ weight measurements and macroscopic and microscopic examinations.

At the end of 13 weeks, weekly measurement of body weight, food and water intake and food utilization revealed a significantly decreased food intake in males and females maintained on the 2949 mg/kg diet and a significantly increased water intake in males at 492 mg/kg and females at 983 mg/kg when compared with controls. In the absence of any dose–response relationship, these changes were not regarded as being related to treatment. There were no treatment-related effects on urine refractive index, urine glutamic–oxaloacetic transaminase levels or qualitative urine analysis (pH, protein, glucose and blood) at 6 or 13 weeks. There were no treatment-related effects on clinical chemistry measurements at 6 or 13 weeks. There were no treatment-related effects on serum protein electrophoresis (albumin, globulin and fibrinogen) at 6 or 13 weeks. While rats administered 492, 983 or 2949 mg/kg of the flavour cocktail for 13 weeks exhibited no significant changes in haematological parameters when compared with controls, rats exposed to 3932 mg/kg of the flavour cocktail for 6 weeks exhibited significantly increased packed cell volume and white blood cell counts compared with controls. Additionally, rats fed 197 mg/kg of the flavour cocktail for 6 weeks showed significantly lower packed cell volume compared with controls. These observations were not considered to be related to the administration of the flavour cocktail.

In animals treated for 6 weeks only, organ weight measurements revealed a statistically significant increase ($P < 0.05$) in relative liver weight in females at the two highest dietary levels (1966 and 3932 mg/kg) compared with controls. The relative liver weight increase observed in treated female rats was slight, not consistent with the results of the 6-week study and not considered to be biologically significant. Significant decreases in absolute brain weights were observed in male rats administered the two highest dietary levels of the flavour cocktail for 6 weeks and in the absolute adrenal weights of all 6-week treated rats; however, there were no significant differences in relative brain and adrenal weights noted between control and treated rats. In the absence of a dose–response relationship, statistically significant effects observed in final mean body and heart weights of male rats as well as in mean heart and spleen weights of female rats were considered not to be treatment related. There was no evidence of gross or histopathological alterations to the liver or any other organs examined in rats of the 6-week study.

In animals treated for 13 weeks, organ weight measurement (i.e. heart, liver, kidneys, spleen, brain, adrenals and testes) revealed a statistically significant increase ($P < 0.05$) in absolute kidney weights of male and female rats at the 1966 and 3932 mg/kg dietary levels and in relative kidney weights of male rats at the 1966 mg/kg level and female rats at the 1966 and 3932 mg/kg dietary levels. Absolute and relative liver weights of males and females administered 197, 1966 or 3932 mg/kg of the flavour cocktail were increased; however, the increases were not marked and were not confirmed by any evidence of liver histopathology. Histopathological examination of the remaining organs showed no evidence of alterations that could be associated with administration of the flavour cocktail. The Committee concluded that no adverse effects could be attributed to treatment with the flavour cocktail (Munday & Kirkby, 1971b).

In a 1-year study, groups of four male and four female rats were maintained on diets containing 197, 492, 983, 1966, 2949 or 3932 mg/kg of a flavour cocktail (equivalent to approximately 0.09, 0.2, 0.4, 0.9, 1.3 and 1.8 mg 2-acetyl-2-thiazoline/kg bw per day, respectively) for a period of 13 weeks, as discussed above (Munday & Kirkby, 1971b). After this 13-week period, all treatment groups (24 males and 24 females) were subsequently maintained on the highest dietary level of the flavour cocktail (3932 mg/kg) from week 15 of the study, for an additional 37 weeks. Final mean body weights and haematological parameter evaluation revealed no significant differences between test and control animals at week 15 and the end of the 1-year study period. No compound-related effects on general health or survival were noted in any of the treated rats. At necropsy, there was no significant difference in absolute or relative organ weights (liver, spleen, heart, kidneys, brain, adrenals, pituitary, thyroid and testes) between treated and control animals. Gross and histopathological examination of all test and control rats revealed no significant macroscopic findings at the end of the 1-year study period. Upon microscopic examination, lesions (subcutaneous sarcoma, chloroma, pituitary adenoma and parafollicular thyroid adenoma) were observed in treated and control rats. Similar findings have been reported in previous studies using control rats of the same strain, and these lesions were considered not to be related to the administration of the flavour cocktail. Dietary administration of the flavour cocktail at a dose of up to 3932 mg/kg (approximately equivalent to 1.8 mg 2-acetyl-2-thiazoline/kg bw per day) for 1 year produced no treatment-related effects on tumour incidence in Colworth Wistar rats (Munday & Kirkby, 1973).

(ii) Thiophene (structurally related substance)

In a 42-day repeat-dose study, groups of Sprague-Dawley rats (13 per sex per dose) were administered, via gavage, 0, 25, 100 or 400 mg thiophene/kg bw per day. The rats were mated, and the reproductive portion of the study is discussed below, under "Developmental and reproductive toxicity". In male and female rats receiving 100 or 400 mg thiophene/kg bw per day, the authors reported leaning (thought to indicate loss of balance) immediately following test article administration. Male and female rats in the 400 mg/kg bw per day group showed a suppression of food consumption and a corresponding suppression of body weight gain compared with controls. Ataxia was observed in females in the 400 mg/kg bw group, and one female was euthanized on becoming moribund on day 8. Clinical chemistry revealed decreases in glucose levels and alkaline phosphatase activity and an increase in inorganic phosphorus levels for males in the 100 and 400 mg/kg bw per day groups. In males, relative liver weights were observed to be increased at dose levels of 100 mg/kg bw per day and greater compared with controls. Increased relative kidney weights and decreased spleen weights were also observed in males administered 100 and 400 mg thiophene/kg bw per day. Additionally, at the 400 mg/kg bw per day dose level, slight increases in eosinophilic bodies were observed in the renal tissue of males. In females, increases in relative liver and kidney weights were observed in the 400 mg/kg bw per day group, whereas vacuolar degeneration of the tubular epithelium was reported at dose levels of 100 mg/kg bw per day and greater. In males, one animal in the 400 mg/kg bw per day group exhibited pyknosis/necrosis of granular cells in the cerebellum. Cerebellar toxicity was also observed

in females, with necrosis of granular cells occurring at thiophene dose levels of 100 mg/kg bw per day and greater. At 400 mg/kg bw per day, female animals also displayed dilatation and oedema of the ventricles. At necropsy, liver changes were noted in both sexes of rats treated with 100 or 400 mg thiophene/kg bw per day. Hepatic variations included hypertrophy of the hepatocytes and infiltration of macrophages into the central zone at the 100 mg/kg bw per day dose level and necrosis and homogenous or vesicular cytoplasmic changes of the hepatocytes in the central zones at the 400 mg/kg bw per day dose level. The NOEL was 25 mg/kg bw per day (Nagao, 2006).

(c) Genotoxicity

(i) Thiophene (structurally related substance)

The results of several in vitro tests for genotoxicity conducted with thiophene, a structurally related compound, are described below.

No increase in mutagenic activity was observed in the reverse mutation assay (Ames test) in *Salmonella typhimurium* strains TA98, TA100, TA1535 or TA1537 at 0, 78.1, 156, 313, 625, 1250, 2500 or 5000 µg thiophene/plate with and without metabolic activation. Toxicity was noted at 1500 µg/plate in TA1537 and at 2500 µg/plate in TA98, TA100 and TA1535 (Shibuya, 2006).

Similarly, there was no increase in mutagenic activity in a mutation assay in *Escherichia coli* strain WP2uvrA at 0, 313, 625, 1250, 2500 or 5000 µg/plate with and without metabolic activation. Toxicity was noted at the 5000 µg/plate concentration (Shibuya, 2006).

There was no increase in chromosomal aberrations or polyploidy following incubation of Chinese hamster lung cells with 0, 210, 420 or 840 µg thiophene/ml (Tanaka, 2006).

(d) Developmental and reproductive toxicity

(i) Thiophene (structurally related substance)

In a 42-day repeat-dose study, groups of Sprague-Dawley rats (13 per sex per dose) were administered, via gavage, 0, 25, 100 or 400 mg thiophene/kg bw per day. There were no adverse effects on copulation, ovulation or fertility in treatment groups as compared with control groups. Abnormal parturition was observed in all treatment groups. Dams in the 100 or 400 mg/kg bw per day group, displaying histopathological changes in the cerebellum, exhibited abnormal lactation. Reduced birth weights, as well as decreased body weights and viability at postnatal day 4, were observed in pups born to dams in the 400 mg/kg bw per day group. There were no visible morphological abnormalities attributed to thiophene administration. The NOEL with respect to maternal toxicity was 25 mg/kg bw per day (Nagao, 2006).

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ALIPHATIC AND AROMATIC AMINES AND AMIDES (addendum)

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 12 flavouring agents, including 1 aliphatic amine (No. 1771); 6 aliphatic amides (Nos 1772–1776 and 1779), 4 of which contain ethanolamine (Nos 1772–1775) and 2 of which contain ring structures (Nos 1776 and 1779); and 5 aromatic amides (Nos 1767–1770 and 1777), 3 of which contain oxalamide (Nos 1768–1770). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, Introduction) (Annex 1, reference *131*). None of these flavouring agents has been evaluated previously by the Committee.

The Committee previously evaluated 37 other members of this group of flavouring agents at its sixty-fifth meeting (Annex 1, reference *178*). The findings presented in that report were considered in the present evaluation. Thirty-six of the

37 substances in that group were concluded to be of no safety concern based on currently estimated levels of intake. However, for 27 of the 36 agents, the intakes were estimated based on anticipated annual volumes of production; as such, the evaluations were deemed conditional pending submission of use levels or poundage data prior to December 2007. One substance, acetamide (No. 1592), was considered inappropriate for use as a flavouring agent or for food additive purposes, based on the available data that indicated that it is clearly carcinogenic in mice and rats. Therefore, the Committee did not evaluate this substance according to the Procedure.

Four of the 12 flavouring agents in this current group (Nos 1771, 1772, 1774 and 1777) have been reported to occur naturally in foods. They have been detected in white wine, spinach, potatoes, sweet potatoes, yams, kale, brown rice, brown rice germ, brown rice sprouts, barley, barley sprouts, beans, bean sprouts, corn, oatmeal, squash, carrot, onion, chestnut, apple, shiitake mushrooms, green laver, lactobacilli and broccoli (Cutillo et al., 2003; Oh et al., 2003; Anon., 2004). No quantitative data on the natural levels in food were available, and therefore consumption ratios (the ratios of their consumption from natural food sources to their use as flavouring agents) were not calculated.

1.2 Assessment of dietary exposure

The total annual volume of production of this group of 12 aliphatic and aromatic amines and amides is approximately 785 kg in the United States of America (USA) and 0.4 kg in Europe. There were no reported uses of these flavouring agents in Japan (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006). In the USA, greater than 91% of the total production volume is accounted for by *N*-gluconyl ethanolamine (No. 1772), *N*-lactoyl ethanolamine (No. 1774), *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) and *N*-3,7-dimethyl-2,6-octadienyl cyclopropylcarboxamide (No. 1779). The estimated per capita intakes in the USA of *N*-gluconyl ethanolamine, *N*-lactoyl ethanolamine, *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide and *N*-3,7-dimethyl-2,6-octadienyl cyclopropylcarboxamide are 13, 10, 34 and 31 µg/day, respectively. The estimated daily per capita intakes in the USA and Europe of all flavouring agents in the group are reported in Table 1. The annual volume of production of each flavouring agent in this group is reported in Table 2.

1.3 Absorption, distribution, metabolism and elimination

The metabolism of aliphatic and aromatic amines and amides was previously described in the report of the sixty-fifth meeting (Annex 1, reference *178*).

In general, aliphatic and aromatic amines and amides are rapidly absorbed in the gastrointestinal tract and transformed to polar metabolites that are readily eliminated in the urine. Many amines are endogenous and have been identified as normal constituents of urine in humans, including 4-aminobutyric acid (No. 1771).

Table 1. Summary of the results of safety evaluations of aliphatic and aromatic amines and amides used as flavouring agents[a,b,c]

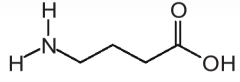
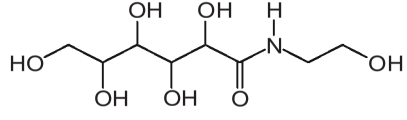
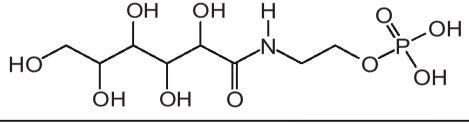
Flavouring agent	No.	CAS No. and structure	*Step A3/B3*[i] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class I						
4-Aminobutyric acid	1771	56-12-2 	No Europe: ND USA: 0.1 Japan: ND	NR	See note 1	No safety concern
N-Gluconyl ethanolamine	1772	686298-93-1 	No Europe: ND USA: 13 Japan: ND	NR	See note 2	No safety concern
N-Gluconyl ethanolamine phosphate	1773	791807-20-0 	No Europe: ND USA: 3 Japan: ND	NR	See note 2	No safety concern

Table 1 (contd)

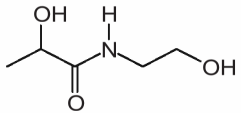
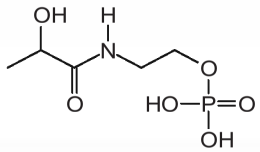
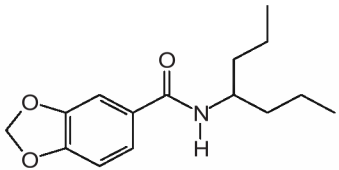
Flavouring agent	No.	CAS No. and structure	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
N-Lactoyl ethanolamine	1774	5422-34-4 	No Europe: ND USA: 10 Japan: ND	NR	See note 2	No safety concern
N-Lactoyl ethanolamine phosphate	1775	782498-03-7 	No Europe: ND USA: 5 Japan: ND	NR	See note 2	No safety concern
Structural class III						
N-(Heptan-4-yl)benzo-[d][1,3]dioxole-5-carboxamide	1767	745047-51-2 	No Europe: 0.01 USA: 0.1 Japan: ND	Yes. The NOEL of 20 mg/kg bw per day (Kot, 2005a) is 10 million times the estimated daily intake of *N*-(heptan-4-yl)benzo-[d][1,3]dioxole-5-carboxamide when used as a flavouring agent.	See note 2	No safety concern

Table 1 (contd)

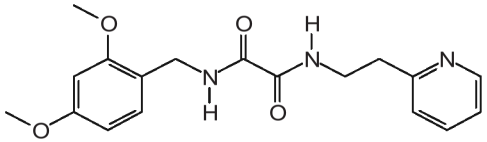
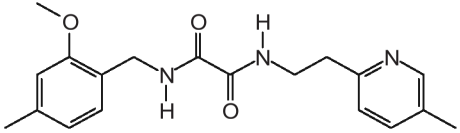
Flavouring agent	No.	CAS No. and structure	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	1768	745047-53-4 	No Europe: 0.01 USA: 0.2 Japan: ND	Yes. The NOEL of 100 mg/kg bw per day (Kot, 2005b) is >33 million times the estimated daily intake of *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide when used as a flavouring agent.	See note 2	No safety concern
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(5-methylpyridin-2-yl)ethyl)oxalamide	1769	745047-94-3 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 100 mg/kg bw per day for the related substance *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) (Kot, 2005b) is 500 million times the estimated daily intake of *N*1-(2-methoxy-4-methylbenzyl)-*N*2-(2-(5-methylpyridin-2-yl)ethyl)-oxalamide when used as a flavouring agent.	See note 2	No safety concern

Table 1 (contd)

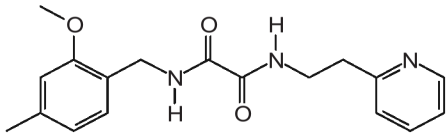
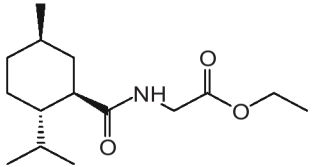
Flavouring agent	No.	CAS No. and structure	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)-oxalamide	1770	745047-97-6 	No Europe: 0.01 USA: 0.01 Japan: ND	Yes. The NOEL of 100 mg/kg bw per day for the related substance *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) (Kot, 2005b) is 500 million times the estimated daily intake of *N*1-(2-methoxy-4-methylbenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide when used as a flavouring agent.	See note 2	No safety concern
N-[(Ethoxycarbonyl)-methyl]-*p*-menthane-3-carboxamide	1776	68489-14-5 	No Europe: ND USA: 34 Japan: ND	Yes. The NOEL of 8 mg/kg bw per day for the related substance *N*-ethyl 2-isopropyl-5-methylcyclohexanecarboxamide (No. 1601) (Miyata, 1995) is >13 000 times the estimated daily intake of *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide when used as a flavouring agent.	See note 3	No safety concern

Table 1 (contd)

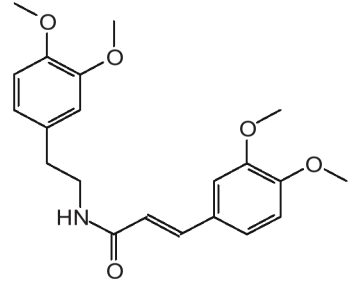
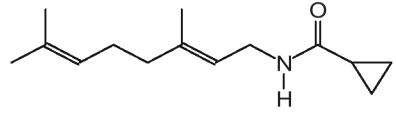
Flavouring agent	No.	CAS No. and structure	*Step A3/B3*[d] Does intake exceed the threshold for human intake?	*Step B4* Adequate margin of safety for the flavouring agent or related substance?	Comments on predicted metabolism	Conclusion based on current intake
N-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide	1777	69444-90-2 	No Europe: ND USA: 0.02 Japan: ND	Yes. The NOEL of 8.36 mg/kg bw per day for the related substance *N*-nonanoyl-4-hydroxy-3-methoxybenzylamide (No. 1599) (Posternak et al., 1969) is >400 000 times the estimated daily intake of *N*-[2-(3,4-dimethoxyphenyl)-ethyl]-3,4-dimethoxycinnamic acid amide when used as a flavouring agent.	See note 2	No safety concern
N-3,7-Dimethyl-2,6-octadienyl cyclopropyl-carboxamide	1779	744251-93-2 	No Europe: ND USA: 31 Japan: ND	Yes. The NOEL of 92 mg/kg bw per day (Merkel, 2005) is >180 000 times greater than the estimated daily intake of *N*-3,7-dimethyl-2,6-octadienyl cyclopropylcarboxamide when used as a flavouring agent.	See note 2	No safety concern

Table 1. (contd)

CAS, Chemical Abstracts Service; ND, no data reported; NR, not required because consumption of the substance was determined to be of no safety concern at Step A3/B3 of the procedure.

[a] Thirty-six flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *178*).

[b] *Step 1:* Five flavouring agents are in structural class I, and seven flavouring agents are in structural class III.

[c] *Step 2:* Five of the agents in this group (Nos 1771–1775) can be predicted to be metabolized to innocuous products. The remaining 7 agents (Nos 1767–1770, 1776, 1777 and 1779) cannot be predicted to be metabolized to innocuous products.

[d] The thresholds for human intake for structural classes I and III are 1800 and 90 µg/person per day, respectively. All intake values are expressed in µg/day.

Notes:

1. Endogenous in mammals and is readily utilized in known metabolic pathways.
2. Amides are subject to limited hydrolysis, with the corresponding ammonium ion or amines entering into known pathways of metabolism and excretion.
3. Anticipated to undergo hydrolysis at the ester moiety followed by conjugate formation and subsequent elimination in the urine.

Table 2. Annual volumes of production of aliphatic and aromatic amines and amides used as flavouring agents in Europe, the USA and Japan

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Intake[b]	
		µg/person per day	µg/kg bw per day
N-(Heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide (1767)			
Europe	0.1	0.01	0.0002
USA	1	0.1	0.002
Japan	ND	ND	ND
*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (1768)			
Europe	0.1	0.01	0.0002
USA	2	0.2	0.003
Japan	ND	ND	ND
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(5-methylpyridin-2-yl)ethyl)oxalamide (1769)			
Europe	0.1	0.01	0.0002
USA	0.1	0.01	0.0002
Japan	ND	ND	ND
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (1770)			
Europe	0.1	0.01	0.0002
USA	0.1	0.01	0.0002
Japan	ND	ND	ND
4-Aminobutyric acid (1771)			
Europe	ND	ND	ND
USA	1	0.1	0.002
Japan	ND	ND	ND
N-Gluconyl ethanolamine (1772)			
Europe	ND	ND	ND
USA	110	13	0.2
Japan	ND	ND	ND
N-Gluconyl ethanolamine phosphate (1773)			
Europe	ND	ND	ND
USA	24	3	0.05
Japan	ND	ND	ND

Table 2 (contd)

Flavouring agent (No.)	Most recent annual volume of production (kg)[a]	Intake[b]	
		µg/person per day	µg/kg bw per day
N-Lactoyl ethanolamine (1774)			
Europe	ND	ND	ND
USA	78	10	0.2
Japan	ND	ND	ND
N-Lactoyl ethanolamine phosphate (1775)			
Europe	ND	ND	ND
USA	37	5	0.08
Japan	ND	ND	ND
N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (1776)			
Europe	ND	ND	ND
USA	278	34	0.6
Japan	ND	ND	ND
N-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (1777)			
Europe	ND	ND	ND
USA	4	1	0.02
Japan	ND	ND	ND
N-3,7-Dimethyl-2,6-octadienyl cyclopropylcarboxamide (1779)			
Europe	ND	ND	ND
USA	250	31	0.5
Japan	ND	ND	ND
Total			
Europe	0.4		
USA	785		
Japan	ND		

bw, body weight; ND, no data reported.

[a] From European Flavour and Fragrance Association (2005), Flavor and Extract Manufacturers Association (2006) and Japanese Flavor and Fragrance Manufacturers Association (2002). Total poundage values of <1 kg reported in the surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006) have been truncated to one place following the decimal point (0.1 kg).

[b] Intake (µg/person per day) calculated as follows:

Table 2. (contd)

[(annual volume, kg) × (1×10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, "consumers only") = 32×10^6 for Europe, 28×10^6 for the USA and 13×10^6 for Japan; and where correction factor = 0.8 for surveys in Europe, the USA and Japan, representing the assumption that only 80% of the annual flavour volume was reported in the poundage surveys (Japanese Flavor and Fragrance Manufacturers Association, 2002; European Flavour and Fragrance Association, 2005; Flavor and Extract Manufacturers Association, 2006).
Intake (μg/kg bw per day) calculated as follows:
(μg/person per day)/body weight, where body weight = 60 kg. Slight variations may occur as a result of rounding.

Aliphatic amides have been reported to undergo hydrolysis in mammals; however, the rate of hydrolysis is dependent on the chain length and may involve a number of different enzymes (Bray et al., 1949). In relation to the substances in this group of flavouring agents, there is only limited information regarding metabolic pathways for specific substances. The four amides containing ethanolamine (Nos 1772–1775) are hydrolysed mainly in the liver, with only minimal hydrolysis occurring in the stomach or intestines (Croonenborgh, 2005a). An *N*-acylethanolamine amidohydrolase has been identified in rat liver that catalyses the hydrolysis of long-chain *N*-acylethanolamines with high efficiency and shorter-chain analogues with lower efficiency (Schmid et al., 1985). *N*-Gluconyl ethanolamine phosphate (No. 1773) and *N*-lactyl ethanolamine phosphate (No. 1775) are simple phosphate esters that are expected to undergo rapid hydrolysis.

N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) is hydrolysed in pancreatic juice and rat liver homogenate, but the major route is ester hydrolysis rather than amide hydrolysis (Poet et al., 2005). With *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767), there was rapid metabolism in the presence of rat hepatocytes, but no amide hydrolysis products were found. A similar result was found with *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)-oxalamide (No. 1768): rapid metabolism in the presence of rat hepatocytes was observed, and no amide hydrolysis products were found (Denning et al., 2004a).

No information is available on the metabolism of the other substances in this group of flavouring agents.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned five flavouring agents (Nos 1771–1775) to structural class I and the remaining seven agents (Nos 1767–1770, 1776, 1777 and 1779) to structural class III (Cramer et al., 1978).

Step 2. Five flavouring agents in this group (Nos 1771–1775) are predicted to be metabolized to innocuous products. The evaluation of these substances

therefore proceeded via the A-side of the Procedure. For the remaining substances, there are limited metabolic data available, and they could not be predicted to be metabolized to innocuous products. The evaluation of these seven flavouring agents (Nos 1767–1770, 1776, 1777 and 1779) therefore proceeded via the B-side of the Procedure.

Step A3. The estimated daily per capita exposures for all five flavouring agents in structural class I are below the threshold of concern (i.e. 1800 µg/person per day for class I). According to the Procedure, these five flavouring agents raise no safety concern when they are used at their currently estimated levels of intake.

Step B3. The estimated daily per capita exposures for the seven flavouring agents in structural class III are below the threshold of concern (i.e. 90 µg/person per day for class III). Accordingly, the evaluation of all seven flavouring agents proceeded to Step B4.

Step B4. For *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767), the no-observed-effect level (NOEL) of 20 mg/kg bw per day from a 93-day study in rats (Kot, 2005a) provides an adequate margin of safety (>10 million) in relation to the currently estimated level of exposure from its use as a flavouring agent in Europe (0.0002 µg/kg bw per day) and in the USA (0.002 µg/kg bw per day).

For *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768), the NOEL of 100 mg/kg bw per day from a 93-day study in rats (Kot, 2005b) provides an adequate margin of safety (>33 million) in relation to the currently estimated level of exposure from its use as a flavouring agent in Europe (0.0002 µg/kg bw per day) and in the USA (0.003 µg/kg bw per day). This NOEL is appropriate for the structurally related flavouring agents *N*1-(2-methoxy-4-methylbenzyl)-*N*2-(2-(5-methylpyridin-2-yl)ethyl)oxalamide (No. 1769) and *N*1-(2-methoxy-4-methylbenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1770), because they are also oxalamides and are expected to be metabolized by similar pathways. For these structurally related flavouring agents, the NOEL of 100 mg/kg bw per day provides a margin of safety of 500 million in relation to the currently estimated levels of exposure to these flavouring agents in both Europe and the USA (0.0002 µg/kg bw per day).

For *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776), the NOEL of 8 mg/kg bw per day for the structurally related substance *N*-ethyl 2-isopropyl-5-methylcyclohexanecarboxamide (No. 1601) from a 28-day study in rats (Miyata, 1995) provides an adequate margin of safety (>13 000) in relation to the currently estimated level of exposure from its use as a flavouring agent in the USA (0.6 µg/kg bw per day).

For *N*-[2-(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777), the NOEL of 8.36 mg/kg bw per day for the structurally related *N*-nonanoyl-4-hydroxy-3-methoxybenzylamide (No. 1599) from a 90-day study in rats (Posternak et al., 1969) provides an adequate margin of

safety (>400 000) in relation to the currently estimated level of exposure from its use as a flavouring agent in the USA (0.02 µg/kg bw per day).

For *N*-3,7-dimethyl-2,6-octadienylcyclopropylcarboxamide (No. 1779), the NOEL of 92 mg/kg bw per day from a 28-day study in rats (Merkel, 2005) provides an adequate margin of safety (>180 000) in relation to the currently estimated level of exposure from its use as a flavouring agent in the USA (0.5 µg/kg bw per day).

Table 1 summarizes the evaluations of the 12 aliphatic and aromatic amines and amides in this group.

1.5 Consideration of combined intakes from use as flavouring agents

The flavouring agents in this group are predicted to be metabolized by hydrolysis, by oxidative metabolism of amino groups and/or aromatic or heterocyclic rings and/or alkyl side-chains and possibly by conjugation with glucuronic acid. Such metabolic pathways have a high capacity and would not be saturated, even if all flavouring agents were consumed at the same time. A number of the substances in this group that have been evaluated at this meeting and at the sixty-fifth meeting contain a primary aliphatic amino group (RCH_2NH_2, where R is an alkyl or aryl structure). Such compounds (Nos 1579, 1580, 1582, 1585 and 1588) are predicted to be metabolized by monoamine oxidase. Trimethylamine oxide (No. 1614) is a metabolite of trimethylamine (No. 1610), and *N*-gluconyl ethanolamine (No. 1772) and *N*-lactoyl ethanolamine (No. 1774) are likely metabolites of the corresponding phosphate esters (Nos 1773 and 1775, respectively), with ethanolamine as a possible minor metabolite of all four substances. The combined intakes of the related substances are below the relevant class I threshold. The other substances in this group have diverse structures, with various potential sites of metabolism, and are not likely to be metabolized to common products. The Committee concluded that under the conditions of use as flavouring agents, the combined intake of the substances in this group would not saturate metabolic pathways and the combined intakes would not raise safety concerns.

1.6 Consideration of secondary components

Two members of this group of flavouring agents, *N*-lactoyl ethanolamine (No. 1774) and *N*-lactoyl ethanolamine phosphate (No. 1775), have assay values of <95%. Information on the safety of the secondary components of these two compounds is summarized in Annex 5 (Summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%). The secondary component of *N*-lactoyl ethanolamine (No. 1774), 2-aminoethanol lactate, is expected to share the same metabolic fate as the main component and is considered not to present a safety concern at the currently estimated levels of exposure. The secondary component of *N*-lactoyl ethanolamine phosphate (No. 1775), ammonium formate, is expected to undergo rapid absorption, distribution, metabolism and excretion from the body and is considered not to present a safety concern at the currently estimated level of exposure.

1.7 Conclusion

In the previous evaluation of substances in this group, studies of acute toxicity, short-term toxicity, long-term toxicity and carcinogenicity, genotoxicity and reproductive toxicity were available. None raised safety concerns. The toxicity data available for this evaluation were supported by those from the previous evaluation.

The Committee concluded that these 12 flavouring agents, which are additions to the aliphatic and aromatic amines and amides evaluated previously, would not give rise to safety concerns at the currently estimated levels of exposure. The Committee noted, while making this conclusion, that 4-aminobutyric acid (No. 1771) is an endogenous neurotransmitter; however, the level in tissues from flavouring use would be insignificant.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

This monograph summarizes key aspects relevant to the safety evaluation of 12 flavouring agents that are additions to the aliphatic and aromatic amines and amides evaluated previously (see Table 1). The group contained one aliphatic amine; six aliphatic amides, four of which contain ethanolamine and two of which contain ring structures; and five aromatic amides, three of which contain oxalamide.

2.2 Additional considerations on intake

There is no additional information on estimated per capita intake.

2.3 Biological data

2.3.1 *Biochemical data: hydrolysis, absorption, distribution, metabolism and excretion*

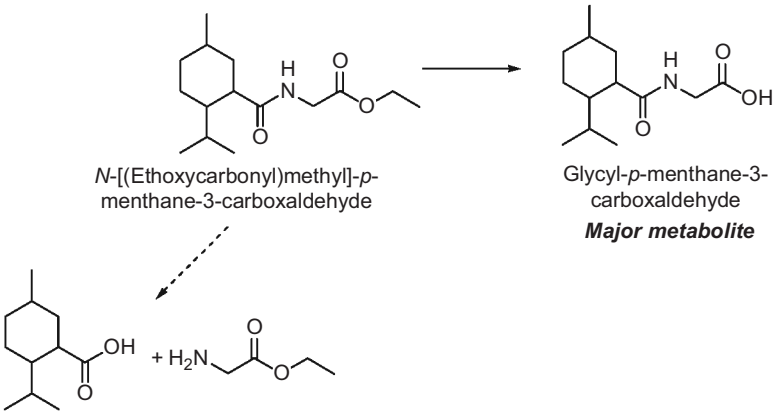
General information on the metabolism of aliphatic and aromatic amines and amides was previously provided in the report of the sixty-fifth meeting (Annex 1, reference *177*). Further information relevant to the substances considered in this report is provided below.

Hydrolysis of ethanolamine amides, such as *N*-gluconyl ethanolamine (No. 1772), *N*-gluconyl phosphate (No. 1773), *N*-lactoyl ethanolamine (No. 1774) and *N*-lactoyl ethanolamine phosphate (No. 1775), is likely to occur only in the liver. In simulated gastric fluids with and without pepsin and in simulated intestinal fluid with and without pancreatin or intestinal pepsidase, *N*-lactoyl ethanolamine (No. 1774) was not hydrolysed (Croonenborgh, 2005b). In a similar study using simulated gastric fluids with and without pepsin and simulated intestinal fluid with and without pancreatin or intestinal pepsidase, *N*-gluconyl ethanolamine (No. 1772) was only partially (<15%) hydrolysed. No difference in the degree of hydrolysis in either medium was observed when enzymes were added (Croonenborgh, 2005a). These results suggest that little hydrolysis of *N*-lactoyl or *N*-gluconyl ethanolamine takes

place in the stomach or intestines. However, an enzyme isolated from rat hepatic tissue, *N*-acylethanolamine amidohydrolase, was shown to be capable of hydrolysing a variety of *N*-acylated ethanolamines (Schmid et al., 1985).

N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) is readily hydrolysed in artificial pancreatic juice and rat liver homogenate (Figure 1). In artificial pancreatic juice containing pancreatin, it was hydrolysed with a half-life of 43.4 ± 14.7 min and a first-order rate constant of 1.06 ± 0.426 per hour, and the rate of hydrolysis was determined to be 0.066 nmol/min per millilitre. In the absence of pancreatin, the half-life increased to >570 min, and the first-order rate constant was reduced to approximately 0.07 per hour. In 5% rat liver homogenate, *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide was rapidly hydrolysed, with a half-life of 0.80 ± 0.19 min. The rates of hydrolysis for 2% and 5% liver homogenates were 0.69 ± 0.14 and 0.83 ± 0.15 nmol/min per milligram protein, respectively. The levels of *p*-menthane-3-carboxylic acid and glycine, the amide hydrolysis products, were lower than would be expected if they were the primary hydrolysis products (Poet et al., 2005). An alternative primary pathway is ester hydrolysis leading to glycyl-*p*-menthane-3-carboxaldehyde and ethanol. Ethyl esters have been shown to be efficiently hydrolysed in rat liver microsomal preparations (Prueksaritanont et al., 1997).

Figure 1. Proposed metabolic pathway for* N-*[(ethoxycarbonyl)methyl]*-p-*menthane-3-carboxamide (No. 1776) by rat liver homogenate



N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxaldehyde

Glycyl-*p*-menthane-3-carboxaldehyde

Major metabolite

p-Menthane-3-carboxylic acid

Ethoxyglycine

Minor metabolites

4-Aminobutyric acid (No. 1771) (γ-aminobutyric acid) is endogenous, as it is formed from decarboxylation of the essential amino acid glutamic acid. It undergoes transamination with α-ketoglutaric acid to re-form glutamic acid and succinic semialdehyde. This semialdehyde is then converted to succinic acid in a reaction

catalysed by the nicotinamide (NAD^+)-dependent aldehyde dehydrogenase (Sierosławska, 1965).

The metabolism of *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767) was investigated in an in vitro hepatocyte model. Subsequent metabolite identification was performed using high-pressure liquid chromatography in conjunction with tandem mass spectrometry (HPLC/MS/MS). The compound was rapidly metabolized in the hepatocyte model to several more polar and conjugated metabolites. Several other potential metabolites were identified with mass differences that did not correspond to any obvious biotransformations. No simple amide hydrolysis metabolites were found. Two main groups of metabolites were formed; the first group included substances resulting from the hydroxylation on the 4-heptamine side-chain, and the other included substances resulting from ring opening of the methylenedioxy group. The 4-heptamine side-chain underwent hydroxylation presumably at the 2-position, which would be analogous to the metabolism of heptane, where hydroxylation occurs primarily at the 2- or 3-position (Perbellini et al., 1986). An additional hydroxylated metabolite was formed, which is likely the 3-position hydroxylation product. A dihydroxylated metabolite was also formed; this metabolite could be the 2,3-dihydroxy derivative, the 2,3′-dihydroxy derivative or some other permutation on these structures (Denning et al., 2004a). The second major group of metabolites centred around the piperonylic acid portion of the molecule, resulting in ring opening or scission of the methylenedioxy bridge system, the formation of the 3,4-dihydroxy derivative and subsequent conjugation (Klungsoyr & Scheline, 1984).

In a similar in vitro rat hepatocyte study, the metabolism of *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) was investigated. The metabolites were identified using HPLC/MS/MS. The compound was rapidly metabolized in the hepatocyte model to several more polar and conjugated metabolites, and, as in the study with *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767), other potential metabolites with mass differences that did not correspond to any obvious biotransformations were also identified. No simple amide hydrolysis metabolites were found. Two main groups of metabolites were formed. The first was a group resulting from demethylation of the ring methoxy groups to form the monohydroxymonomethoxy isomers. The monohydroxy metabolites also underwent glucuronidation (Denning et al., 2004b). The second major group of metabolites likely involved further hydroxylation of the pyridine ring system. 2-Substituted pyridines do not form *N*-oxide derivatives, but they do undergo hydroxylation at the 5- and 6-positions (Hawksworth & Scheline, 1975).

2.3.2 Toxicological studies

(a) Acute toxicity

Oral LD_{50} values have been reported for 3 of the 12 substances in this group. For *N*-3,7-dimethyl-2,6-octadienylcyclopropylcarboxamide (No. 1779) and *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide, the LD_{50} in rats was >2000 mg/kg bw (No. 1776) (Merkel, 2004; Culling, 2005).

For *N*-gluconyl ethanolamine (No. 1772), the LD_{50} in mice was 4000 mg/kg bw (Dubois et al., 1992).

(b) Short-term studies of toxicity

The results of short-term studies of toxicity are summarized in Table 3 and discussed below.

(i) N-*(Heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767)*

In a 93-day subchronic toxicity study, male and female Crl:CD(SD)IGS BR rats (20 per sex per group) were fed a diet providing *N*-(heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide at 0 (basal diet), 2, 10 or 20 mg/kg bw per day. Animal survival was measured, and clinical observations, including ophthalmic examinations, were made. Body weight change, food consumption, haematology, clinical chemistry, urinalysis and organ weights were measured. Immunohistochemical examination was undertaken, as well as macroscopic examination and histopathological evaluation. Rats in the test groups showed no significant differences in mean weekly body weights and food consumption when compared with controls. Although statistically significant differences were observed sporadically in the body weight gain of males at all dose levels in comparison with controls, these differences were infrequent and inconsistent, did not affect the mean body weights and were not

Table 3. Results of short-term studies of toxicity with aliphatic and aromatic amines and amides used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1767	*N*-(Heptan-4-yl)benzo-[d][1,3]dioxole-5-carboxamide	Rat; M, F	3/40	Diet	93	20[c]	Kot (2005a)
1768	*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)-oxalamide	Rat; M, F	3/40	Diet	92 or 93	100[c]	Kot (2005b)
1779	*N*-3,7-Dimethyl-2,6-octadienylcyclopropyl-carboxamide	Rat; M, F	3/10	Diet	28	92 (M)[c] 97 (F)[c]	Merkel (2005)

F, female; M, male.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] Study performed with either a single dose or multiple doses that had no adverse effect; the value is therefore not a true NOEL, but is the highest dose level tested that produced no adverse effects. The actual NOEL may be higher.

considered to be toxicologically significant. Red eye discharge was noted in both males and females at all dose levels. Although lens opacity and chromodacyorrhoea, considered to be related to the red eye discharge, were detected in one control male and two high-dose females, respectively, these observations were not considered to be adverse or necessarily attributable to *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide exposure in the diet. No significant differences were reported in any of the other parameters evaluated in this study, and no gross or microscopic abnormalities related to the administration of *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide were identified. The NOEL was the highest dose tested—namely, 20 mg/kg bw per day (Kot, 2005a).

(ii) N*1-(2,4-Dimethoxybenzyl)*-N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768)*

In a 92- or 93-day subchronic toxicity study, male and female Crl:CD(SD) IGS BR rats (20 per sex per group) were fed a diet providing *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide at 0 (basal diet), 10, 30 or 100 mg/kg bw per day. Animal survival was measured, and clinical observations, including ophthalmic examination, were made. Measurements of body weight change, food consumption, haematology, clinical chemistry, urinalysis and organ weights were undertaken. Bone marrow smears were taken, as well as macroscopic examination and histopathological evaluation. Dietary administration of *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide induced no treatment-related changes in any of these factors. In one high-dose female, diffuse right corneal opacity with linear/streak and temporal failure to dilate the right iris anterior chamber was observed; however, this incidental finding was considered to be unrelated to treatment. In addition, there was a slight but statistically significant decrease observed in the relative heart weight of the 30 mg/kg bw per day male group; however, this was not observed in the higher dose group, there was no supporting histopathology and it was considered to be not related to the dietary administration of the test material. Apart from a variety of spontaneously occurring lesions, there were no histopathological changes in any of the examined tissues that could be attributed to the test compound. The NOEL was the highest dose tested—namely, 100 mg/kg bw per day (Kot, 2005b).

(iii) N-*3,7-Dimethyl-2,6-octadienylcyclopropylcarboxamide (No. 1779)*

A 28-day dietary toxicity study was conducted in male and female CRL:CD (SD) IGS BR rats that were fed a diet containing *N*-3,7-dimethyl-2,6-octadienylcyclopropylcarboxamide at concentrations of 0, 11, 110 or 1100 mg/kg. These dietary levels corresponded to measured daily intakes of 0, 0.92, 9 and 92 mg/kg bw and 0, 0.98, 10 and 97 mg/kg bw for males and females, respectively. Whereas 5 males and 5 females were included at the low- and mid-dose levels, the control and high-dose groups consisted of 10 males and 10 females.

The test and control diets were presented to their respective groups on day 0 of the study. All the low- and mid-dose animals and half of the control and high-dose animals (non-recovery groups 1–8) were exposed to their test or control diet for at least 28 days, prior to necropsy on day 31. The remaining control and high-dose animals (groups 9–12) constituted the recovery group and were exposed to

the test or control diet for 28 days, then were fed unsupplemented feed for an additional 14 days before necropsy on day 45.

Prior to initial dosing and again on day 28, all rats were weighed and examined for visual impairment. In addition, all animals were observed daily for general health, symptoms of toxicity and behavioural changes. All rats were subjected to detailed weekly observation, including body weight and food consumption. Functional observational battery and motor activity tests were performed on test groups 1–8 at week 4 post-initiation and on the recovery group animals at week 6. Blood for haematological and clinical biochemistry analysis was collected from groups 1–8 at week 5 and from groups 9–12 at week 7. Animals providing blood samples were fasted 24 h prior to collection, and a urine sample was also collected from each animal. At the conclusion of the test period, gross necropsies were performed on all study rats, and selected organs and tissues were evaluated histologically in the control and high-dose groups.

There were no test substance–related mortalities or clinical effects. A significant increase in food efficiency was reported during an unspecified measurement interval in low-dose males compared with controls; however, as this was transient and not dose related, it was not considered to be toxicologically significant. Urinalysis revealed no significant findings for any of the test groups as compared with controls. Haematology and clinical biochemistry revealed increases in mean cell volume in the low-dose and non–recovery group high-dose females. Low-dose females also exhibited an increase in mean cell haemoglobin compared with controls. Red blood cell counts for the 1100 mg/kg recovery group females were statistically significantly increased compared with control values, but this occurred towards the end of the recovery period and therefore was not considered to be associated with the administration of the test material. Sorbitol dehydrogenase levels were increased in the 11 mg/kg and non–recovery group 1100 mg/kg males; however, this was attributed to the unusually low levels of sorbitol dehydrogenase in control rats (Everds, 2005; Merkel, 2005). Compared with controls, significant increases in the organ to body weight and the organ to brain weight ratios of the thymus were reported in the high-dose non–recovery group males. In females, a significant increase in the organ to body weight ratio of the liver was reported in the high-dose non–recovery group compared with controls. Macroscopic examination revealed gross lesions of the liver, lung, spleen, uterus, caecum, lymph nodes and kidneys in both sexes of animals and at varying dose levels; however, incidence of these lesions did not reach statistical significance compared with controls. Moreover, there were no underlying microscopic abnormalities associated with any of the lesions, or the microscopic changes were considered to be incidental and unrelated to the presence of the test material in the feed. Given the absence of any microscopic abnormalities related to the dietary administration of the test material, the relative organ weight variations were determined to be clinically irrelevant (Funk, 2005; Merkel, 2005).

Under the conditions of this study and based on the toxicological end-points evaluated, the NOEL was the highest dose tested—namely, 1100 mg/kg, equivalent to 92 and 97 mg/kg bw per day in the non–recovery group male and female rats, respectively, and 94 and 97 mg/kg bw per day in the recovery group male and female rats, respectively (Everds, 2005; Funk, 2005; Merkel, 2005).

(c) Genotoxicity

In vitro and in vivo genotoxicity testing has been performed on six flavouring agents in this group. The results of these studies are summarized in Table 4 and described below.

(i) In vitro

N-(Heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767), *N*-gluconyl ethanolamine (No. 1772), *N*-lactoyl ethanolamine (No. 1774) and *N*-[2-(3,4-dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide (No. 1777) were tested in *Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537 and *Escherichia coli* WP2uvrA at concentrations up to 5000 µg/plate, with and without S9 activation. There was no evidence of an increase in revertants (Uhde, 2004; Verspeek-Rip, 2004a, 2004b; Zhang, 2004a).

*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) induced an increase in the number of revertants in *S. typhimurium* TA1535 in the absence (but not in the presence) of metabolic activation compared with control values; however, no dose–response was observed, and the mean number of revertants was reported to be below historical spontaneous reversion or negative control values. When tested under the conditions of the preincubation assay at concentrations of up to 5000 µg/plate, *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) induced an increase in the number of revertants in *S. typhimurium* TA100 in the presence of metabolic activation, but only at a concentration of 62 µg/plate; no dose–response pattern was observed, and no significant increases in the number of revertants were reported in the absence of metabolic activation at concentrations of up to 5000 µg/plate. Moreover, *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) consistently tested negative in several other strains of *S. typhimurium* (TA98 and TA1537) and in *E. coli* WP2uvrA in both the absence and presence of metabolic activation, in both plate incorporation and preincubation assays, at concentrations of up to 5000 µg/plate. Given the lack of a dose-dependent response, non-reproducibility of results and the fact that the number of revertants was below historical control values, it was concluded that *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) was non-mutagenic (Zhang, 2004a).

Table 4. Studies of genotoxicity with aliphatic and aromatic amines and amides used as flavouring agents

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
In vitro						
1767	*N*-(Heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide	Reverse mutation[a]	*Salmonella typhimurium* TA98, TA100, TA1535 and TA1537	0, 21, 62, 190, 560, 1670 or 5000 μg/plate[b]	Negative[c]	Zhang (2004a)
1767	*N*-(Heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide	Reverse mutation[a]	*Escherichia coli* WP2uvrA	0, 21, 62, 190, 560, 1670 or 5000 μg/plate[b]	Negative[c]	Zhang (2004a)
1767	*N*-(Heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide	Chromosomal aberration	Chinese hamster ovary cells	0, 21, 62, 190, 560, 1670 or 5000 μg/ml	Negative[c]	Zhang (2004b)
1768	*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)oxalamide	Reverse mutation[a]	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0, 21, 62, 190, 560 or 1670 μg/plate	Weakly positive/ negative[c,d]	Zhang (2005a)
1768	*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)oxalamide	Reverse mutation[a]	*E. coli* WP2uvrA	0, 21, 62, 190, 560, 1670 or 5000 μg/plate	Negative[c]	Zhang (2005a)
1768	*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)oxalamide	Chromosomal aberration	Chinese hamster ovary cells	0, 21, 62, 190, 560, 1670 or 5000 μg/ml	Negative[c]	Zhang (2005b)
1772	*N*-Gluconyl ethanolamine	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0, 3,[e] 10,[e] 33,[e] 100, 333, 1000, 3330 or 5000 μg/plate	Negative[c]	Verspeek-Rip (2004a)
1772	*N*-Gluconyl ethanolamine	Reverse mutation	*E. coli* WP2uvrA	0, 3, 10, 33, 100, 333, 1000, 3330 or 5000 μg/plate	Negative[c]	Verspeek-Rip (2004a)
1774	*N*-Lactoyl ethanolamine	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0, 3,[e] 10,[e] 33,[e] 94, 310, 940, 3140 or 4720 μg/plate	Negative[c]	Verspeek-Rip (2004b)

Table 4 (contd)

No.	Flavouring agent	End-point	Test object	Concentration	Results	Reference
1774	*N*-Lactoyl ethanolamine	Reverse mutation	*E. coli* WP2uvrA	0, 3, 10, 33, 94, 310, 940, 3140 or 4720 µg/plate	Negative[c]	Verspeek-Rip (2004b)
1776	*N*-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	0, 50, 150, 500, 1500, 2000,[f] 3000,[f] 4000[f] or 5000 µg/plate	Weakly positive[c,g]	Thompson (2005)
1776	*N*-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide	Reverse mutation	*E. coli* WP2uvrA	0, 50, 150, 500, 1500 or 5000 µg/plate	Negative[c]	Thompson (2005)
1777	*N*-[2-(3,4-Dimethoxy-phenyl)ethyl]-3,4-dimethoxycinnamic acid amide	Reverse mutation[a]	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0, 31.6, 100, 316, 1000 or 3160 µg/plate	Negative[c]	Uhde (2004)
In vivo						
1767	*N*-(Heptan-4-yl)benzo[d][1,3]-dioxole-5-carboxamide	Micronucleus induction	Swiss albino (CD-1) mice	0, 175, 350 or 700 mg/kg bw[h]	Negative	Pucaj (2004a)
1768	*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridine-2-yl)ethyl)oxalamide	Micronucleus induction	Swiss albino (CD-1) mice	0, 200, 400 or 800 mg/kg bw[h]	Negative	Pucaj (2004b)
1772	*N*-Gluconyl ethanolamine	Micronucleus induction	NMRI BR mice	0 or 2000 mg/kg bw[i]	Negative	Buskens (2004a)
1774	*N*-Lactoyl ethanolamine	Micronucleus induction	NMRI BR mice	0 or 2000 mg/kg bw[i]	Negative	Buskens (2004b)

[a] Plate incorporation assay and preincubation assay.

[b] The maximum concentration tested was 1670 µg/plate except for *Salmonella typhimurium* TA100 in the plate incorporation assay, for *S. typhimurium* TA98 and TA100 and *Escherichia coli* WP2uvrA in the preincubation assay without S9 (9000 × *g* supernatant from rat liver) and for *S. typhimurium* TA98, TA1535 and TA1537 and *E. coli* WP2uvrA in the preincubation assay with S9, because of precipitation.

[c] With and without metabolic activation.

Table 4. (contd)

[d] In the plate incorporation assay, *S. typhimurium* TA1535 tested positive at concentrations of 21, 190 and 1670 µg/plate, but only without S9. In the preincubation assay, *S. typhimurium* TA100 tested positive only at 62 µg/plate and only with S9.
[e] For *S. typhimurium* TA100 only.
[f] *S. typhimurium* TA100 and TA1535 tested without S9 using both plate incorporation and preincubation methods.
[g] Weak incidence of reverse mutation observed only in *S. typhimurium* TA100 and TA1535. All other tester strains showed no evidence of mutagenicity.
[h] Test material administered via single intraperitoneal injection.
[i] Test material administered via single gavage dose.

N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) induced a slight increase in the number of revertants in *S. typhimurium* TA100 and TA1535 in the absence of metabolic activation compared with control values; however, the increase was not statistically significant. In a set of confirmatory experiments, *S. typhimurium* TA100 and TA1535 were retested at concentrations of 0, 2000, 3000, 4000 or 5000 µg/plate without metabolic activation. The study reported an increase in revertant colonies in strain TA1535 that was reproducible and, at the highest concentration tested, was significantly above in-house historical controls. The report concluded that *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) was weakly mutagenic to TA1535 under the test conditions. In contrast, increases observed in the revertant colonies in *S. typhimurium* TA100, although statistically significant, were small and did not follow a dose–response pattern. Moreover, *N*-[(ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide (No. 1776) consistently tested negative in several other strains of *S. typhimurium* (TA98 and TA1537) and in *E. coli* WP2uvrA in both the absence and presence of metabolic activation, at concentrations of up to 5000 µg/plate (Thompson, 2005).

N-(Heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767) and *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768) produced no evidence of genotoxicity at 0, 21, 62, 190, 560, 1670 or 5000 µg/ml in standard chromosomal aberration assays in Chinese hamster ovary cells cultured with and without S9 metabolic activation (Zhang, 2004b, 2005b).

(ii) In vivo

In a standard mouse micronucleus bone marrow assay, groups of 21 male Swiss albino (CD-1) mice per dose were injected intraperitoneally with 0, 175, 350 or 700 mg *N*-(heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide (No. 1767)/kg bw. At 24, 36 and 48 h following dose administration, seven mice from each group were killed, and their femoral bone marrow was harvested, fixed and stained. No statistically significant differences were observed in the number of polychromatic erythrocytes with micronuclei between the test groups and the negative control (Pucaj, 2004a).

In a standard mouse micronucleus bone marrow assay using the same protocol as described above, groups of 21 male Swiss albino (CD-1) mice per dose were injected intraperitoneally with 0, 200, 400 or 800 mg *N*1-(2,4-dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide (No. 1768)/kg bw. At 24, 36 and 48 h following dose administration, seven mice from each group were killed, and their femoral bone marrow was harvested, fixed and stained. No statistically significant differences were observed in the number of polychromatic erythrocytes with micronuclei between the test groups and the negative control (Pucaj, 2004b).

In a similar standard mouse micronucleus bone marrow assay, male NMRI BR mice (five per group) were administered aqueous *N*-gluconyl ethanolamine (No. 1772) at 0 (negative or positive control) or 2000 mg/kg bw via gavage. Femoral bone marrow was isolated at 24 or 48 h post-administration. Treatment and control mice showed no difference in the ratio of polychromatic to normochromatic erythrocytes.

N-Gluconyl ethanolamine (No. 1772) showed no mutagenic potential in the mouse micronucleus assay (Buskens, 2004a).

Employing the same standard mouse micronucleus bone marrow assay as used above, male NMRI BR mice (five per group) were administered aqueous *N*-lactoyl ethanolamine (No. 1774) at 0 (negative or positive control) or 2000 mg/kg bw via gavage. Femoral bone marrow was isolated 24 or 48 h after administration. Treated and control mice showed no difference in the ratio of polychromatic to normochromatic erythrocytes. *N*-Lactoyl ethanolamine (No. 1774) showed no mutagenic potential in the mouse micronucleus assay (Buskens, 2004b).

(iii) Conclusion for genotoxicity

On the weight of evidence, negative results were obtained with the flavouring agents of this group when tested in in vitro mutation assays in *S. typhimurium* and *E. coli*, as well as in mammalian cells. Negative results were also obtained in in vivo micronucleus assays.

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CONTAMINANTS

AFLATOXINS: IMPACT OF DIFFERENT HYPOTHETICAL LIMITS FOR ALMONDS, BRAZIL NUTS, HAZELNUTS, PISTACHIOS AND DRIED FIGS

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1. EXPLANATION

The aflatoxins (AFL) were evaluated by the Committee at its thirty-first, forty-sixth, forty-ninth and fifty-sixth meetings (Annex 1, references *77*, *122*, *131* and *152*). At the thirty-first meeting, the Committee considered AFL to be a potential human carcinogen and urged that dietary exposure to AFL be reduced to the lowest practicable levels, so as to reduce the potential risk as far as possible. The International Agency for Research on Cancer also concluded that naturally occurring AFL are carcinogenic to humans. At its forty-sixth meeting, the Committee considered estimates of the carcinogenic potency of AFL and the potential risk associated with their intake. In view of the value of such estimates, the Committee recommended that this task be continued at a subsequent meeting. At its forty-ninth meeting, the Committee analysed the effects of applying hypothetical standards for contamination in maize and groundnuts with AFL B_1 (AFB_1; 10 and 20 µg/kg) and concluded that reducing the standard from 20 to 10 µg/kg would not result in any observable difference in the rates of liver cancer. At its fifty-sixth meeting, the Committee concluded that the potency of AFL in hepatitis B virus surface antigen positive ($HBsAg^+$) individuals is substantially higher than the potency in hepatitis B virus surface antigen negative ($HBsAg^-$) individuals and that the liver cancer burden could best be reduced by giving priority to vaccination campaigns against hepatitis

B and to prevention of infection with hepatitis C; the latter would require greater control of blood and blood products.

The Codex Committee on Food Additives and Contaminants at its thirty-eighth session (Codex Alimentarius Commission, 2006) requested that the Committee conduct a dietary exposure assessment for total aflatoxins (AFT) from consumption of tree nuts (ready-to-eat)—in particular, almonds, Brazil nuts, hazelnuts and pistachios—and analyse the impact on dietary exposure of hypothetical maximum limits (MLs) of 4, 8, 10 and 15 µg/kg with consideration of the overall dietary AFT exposure, including consumption of maize and groundnuts. An additional request was received by the Committee to take into account in its assessment an additional hypothetical ML of 20 µg/kg.

In this evaluation, the sum of AFL B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2) is referred to as AFT. The Committee agreed that this assessment applies to the edible parts of almonds (Codex food and feed classification number TN 0660) of cultivars grown from *Prunus amygdalus*, to Brazil nuts (TN 0662) ("white almonds") of the species *Bertholletia excelsa*, to the "common edible hazelnuts" (TN 0666) from *Corylus avellana* intended for direct consumption and to pistachio nuts (TN 0675) of cultivars grown from *Pistacia vera*. Additionally, the evaluation considered dried figs (DF 0297) from ripe fruits of cultivars grown from *Ficus carica* and intended for direct consumption. It does not apply to dried figs intended for processing.

AFL occurrence and concentration data, submitted from 22 European Union (EU) Member States for the European Food Safety Authority (EFSA) risk assessment requested by the European Commission (EC) in 2006, were available for this evaluation. Australia, Brazil, the Islamic Republic of Iran, Japan, Turkey, United Arab Emirates and the United States of America (USA) also submitted data on AFL contamination. In total, the Committee had access to over 100 000 data points for its analyses. Other data on contamination by these toxins have been taken from published literature, but they were not used to calculate dietary exposure because the disaggregated data were not available. Rather, they were used to reinforce the analysis made in the document.

The results of studies relevant to a toxicological evaluation, particularly metabolic and epidemiological studies, published since the last Joint FAO/WHO Expert Committee on Food Additives (JECFA) risk assessment of AFL, did not alter that assessment and indeed lent support to the conclusions reached in that assessment. They were not further considered in this current assessment.

2. ANALYTICAL METHODS

2.1 Chemistry

AFL are a group of related coumarin derivatives; the bifuran nucleus and the pentaheterocyclic arrangement lend rigidity to their structure. There are several known AFL (National Center for Biotechnology Information, 2007), but most of these are metabolites formed endogenously in animals and humans or AFL derivatives

formed during processing. The key AFL present in almonds, Brazil nuts, hazelnuts, pistachios and dried figs are (International Agency for Research on Cancer, 2002; European Food Safety Authority, 2007):

- AFB_1 (Chemical Abstracts Service [CAS] No. 1162-65-8), which has the synonyms 6-methoxydifurocoumarone; 2,3,6aα,9aα-tetrahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione; and (6a*R*-*cis*)-2,3,6a,9a-tetrahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione;
- AFB_2 (CAS No. 7220-81-7), which has the synonyms dihydroaflatoxin B_1; 2,3,6aα,8,9,9aα-hexahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]-benzopyran-1,11-dione; (6a*R*-*cis*)-2,3,6a,8,9,9a-hexahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione; and (6a*R*,9a*S*)-2,3,6a,8,9,9a-hexahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]-benzopyran-1,11-dione (9CI);
- AFG_1 (CAS No. 1165-39-5), which has the synonyms 3,4,7aα,10aα-tetrahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]pyrano-[3,4-*c*][*l*]benzopyran-1,12-dione; (7a*R*-*cis*)-3,4,7a,10a-tetrahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo-[2,3-*h*]pyrano-[3,4-*c*][*l*]benzopyran-1,12-dione; and (7a*R*,10a*S*)-3,4,7a,10a-tetrahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]pyrano[3,4-*c*][*l*]-benzopyran-1,12-dione (9CI);
- AFG_2 (CAS No. 7241-98-7), which has the synonyms dihydroaflatoxin G_1; 3,4,7aα,9,10,10aα-hexahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]-pyrano[3,4-*c*][*l*]benzopyran-1,12-dione; (7a*R*-*cis*)-3,4,7a,9,10,10a-hexahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]-furo[2,3-*h*]pyrano[3,4-*c*][*l*]benzopyran-1,12-dione; and (7a*R*,10a*S*)-3,4,7a,9,10,10a-hexahydro-5-methoxy-1*H*,12*H*-furo-[3′,2′:4,5]furo[2,3-*h*]pyrano[3,4-*c*][*l*]benzopyran-1,12-dione (9CI).

Other information and chemical properties of some naturally occurring AFL and metabolites are included in previous evaluations (International Programme on Chemical Safety, 1979; Annex 1, references *77*, *122*, *131* and *153*).

2.2 Description of analytical methods

AFL occurrence and concentration data, submitted from 22 EU Member States for the EFSA risk assessment requested by the EC in 2006, were available for this evaluation. In the EU, methods of analysis for the official control (enforcement, defence and referee purposes) of the levels of AFL and other mycotoxins in foodstuffs have to fulfil the analytical requirements laid down in Annex II of Commission Regulation EC No. 401/2006 (European Commission, 2006). These include, among others, criteria for laboratory blanks, recovery and precision and specify that the analytical result corrected for recovery shall be used for controlling compliance. Some details of the EU methodology can be found in European Food Safety Authority (2007).

Excellent reviews and descriptions of analytical methods for AFL are available (Gilbert & Anklam, 2002; Gilbert & Vargas, 2003, 2005; AOAC International, 2005; Krska et al., 2005; Krska & Molinelli, 2007). Specific references

on AFL analytical methodology not described in these reviews are, for tree nuts, Chan et al. (2004), Lee et al. (2004), Sapsford et al. (2006) and Aghamohammadi et al. (2007); and for dried figs, Stroka et al. (2000) and Iamanaka et al. (2007).

Although there are several analytical methodologies described for AFL in tree nuts and dried figs, the submitted data were determined using only a few of them (Trucksess et al., 1994; VICAM, 1999; AOAC International, 2000a, 2000b; Ministério da Agricultura, Pecuária e Abastecimento, 2000; Stroka et al., 2000; Akiyama et al., 2001, 2002; R-Biopharm Rhône Ltd, 2002; Neogen, 2007). In the studies evaluated by the Committee at its present meeting, it was usually clear which AFL analytical method had been used.

In all of these methods, AFL are extracted from the samples with organic solvents, acetonitrile or methanol, in combination with small amounts of water. In the case of Brazil nuts or dried figs, instead of water, some samples are extracted with the organic solvent and an aqueous solution with potassium chloride or sodium chloride.

A major problem associated with most analytical methods for the determination of AFL is the presence of co-extractives with the potential to interfere with the analysis. Three different cleanup principles were used to assess AFL contamination in the submitted data. Formerly, liquid–liquid partitioning was the most commonly applied procedure for removing unwanted matrix components in the sample extract. This procedure, however, uses vast amounts of solvents, leads to losses and is time consuming. In the submitted data, only one country employed ammonium sulfate or copper(II) sulfate solutions to precipitate interfering co-extractives before passage through celite and liquid–liquid partitioning with chloroform. Special attention has been given to the reduction of the use of chlorinated solvents by the employment of alternative extractants; as a consequence, new immunological or solid-phase cleanups have been developed. This could be the reason why in the other analytical methodologies, the extract interferences are eliminated, sometimes by passage through multifunctional columns (almonds, hazelnuts and pistachios), through a silica gel column before an immunoaffinity column (dried figs) or directly by immunoaffinity columns for all the matrices.

The extracts are then concentrated, usually by evaporation under nitrogen or vacuum. Afterwards, they are separated by thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) or measured directly on a fluorometer (almonds, hazelnuts and pistachios) or a microwell reader (for all four tree nuts). AFL are visualized by TLC under ultraviolet (UV) light and quantified by visual comparison with known concentrations of standards. The methods using HPLC for AFL analyses are chosen because of their sensitivity and improved accuracy. The differences between methodologies used for the submitted data in this step are the type of detector used (UV light detectors or fluorescence detectors [FLD]) and the timing of derivatization (before or after passing through the HPLC column).

Recoveries of AFL in the different substrates sometimes affect the occurrence data more than the sensitivity of the quantification method. If the

extraction and cleanup steps are effective, it is also possible to obtain low limits of detection (LODs) or limits of quantification (LOQs) with low-cost methods such as TLC. During the JECFA meeting on several mycotoxins in 2001, mycotoxins considered for estimating intake were proposed to have recoveries greater than 60% at the LOQ level. It seems to be relatively easy to attain these levels with AFL, because all the available recoveries in the submitted data were greater than 70%, most of the recoveries being higher than 85%. Small recoveries could lead to underestimates of AFL exposure.

To define the contamination levels, one approach is to correct the results from the recoveries of the analytical methodology (commonly obtained by using one, two or three spiked levels), which may lead to error, as recoveries depend on the different contamination levels. To use this approach, it is necessary to estimate recoveries in more levels within the range of the analytical methodology. The Committee also concluded that it was better to restrict data used in the dietary exposure assessment to those with validated recoveries greater than 70% than to correct for lower recoveries.

It is important to point out that AFL recoveries for the analytical methodology chosen should be determined in the diverse raw matrices as well as in the products obtained from the different processes, as the variation in oil content in tree nuts and the different sugar concentrations in dried figs depend not only on the initial composition of the figs, but also on the drying process and sugar addition.

In the submitted data, LODs and LOQs for AFT were calculated in different ways. One method defined the LOD of AFT as twice the value of the LOD of AFB_1, whereas the second used the sum of the LODs of AFB_1, AFB_2, AFG_1 and AFG_2. The Committee concluded that both definitions overestimate the LOD of AFT, resulting in conservative estimates of the exposure to AFL for the upper-bound estimate.

Quantification limits are sometimes called limits of reporting (LORs) or limits of determination, and detection limit is also called limit of determination. To avoid ambiguity, the following names and definitions are used in this monograph:

- *Limit of detection:* The LOD is the lowest concentration of a chemical that can be qualitatively detected using a specified laboratory method (i.e. its presence can be detected but not quantified).
- *Limit of quantification:* The LOQ is the lowest concentration of a chemical that can be detected and quantified, with an acceptable degree of certainty, using a specified laboratory method.

The Committee also noted that surveillance data should be accompanied by a clear description of the analytical method used; recoveries of the analytical methodology chosen should be specific to the food matrix tested; and LODs and LOQs should be provided with the definitions used to derive them. Efforts should be made to harmonize the nomenclature and the methodologies by which the LOD and LOQ are calculated.

Generally, for accurate exposure assessments, the LOD/LOQ should be as low as technically possible, since most foods will not contain detectable contamination and the value assigned to those samples will affect the estimated exposures. The LODs for AFL varied considerably between laboratories and different foods. The minimum LOD reported for AFB_1 in European Food Safety Authority (2007) was 0.0002 µg/kg, and the maximum LOD was 10 µg/kg, but usually the LOD was reported at around 0.1 µg/kg.

With respect to the definition of the LOD of AFT, other methodological approaches should be tested, such as uniform, normal or lognormal distributions or methods based on quantiles (see, for example, Harter & Moore, 1966; Reid, 1981; Roger & Peacock, 1982; Gilliom & Helsel, 1986; Green & Crowley, 1986; Travis & Land, 1990; Hecht & Honikel, 1995; Vlachonikolis & Marriott, 1995; Giersbrecht & Whitaker, 1998; Korn & Tyler, 2001).

There is an increasing demand for screening techniques with quick and reliable results for field or industrial processors. Most of them are mentioned in the reviews of Gilbert & Vargas (2003) and Krska et al. (2005), although they are not fully validated for the products evaluated in this meeting. Progress was noted on the development of screening techniques for AFL using sometimes quite innovative approaches. Many of these techniques showed promise, such as lateral flow devices; these one-step tests take only 2–3 min to perform (Krska et al., 2005).

Continued progress on the development of improved sample cleanup techniques with good recoveries has been observed. An example of the application of a new cleanup column was an improvement of a fluorometric test kit that determines AFL in almonds, allowing it to be validated by the AOAC International Research Institute as a Performance Tested certified kit (Romer Labs, 2007).

The combination of liquid chromatography with mass spectrometry is one useful technique for the confirmation of the presence of AFL in foodstuffs. The improvement and availability of different types of mass spectrometers, such as quadrupole, ion-trap, time-of-flight instruments and combinations, not only allow the confirmation of the presence of mycotoxins, but will also lead to powerful multiresidue methods for mycotoxin analysis and also multisubstrate methods in the near future (Sulyok et al., 2006; Krska & Molinelli, 2007). A liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometric method is described for the determination of AFB_1, AFB_2, AFG_1 and AFG_2 in figs, but the percentage of AFL recovery from extracts spiked with AFL was lower than that observed in the submitted data using HPLC-FLD (Vahl & Jùrgensen, 1998). As mycotoxins belong to different chemical families and have a broad range of polarities, sample pretreatment is a critical step; otherwise, significant losses may occur during extraction or cleanup. The number and types of mycotoxins analysed and their recoveries will be determined in part by the conditions during sample preparation and chromatographic separation. Although some methods are already implemented in routine analysis, the limited number of reference materials, high investment costs and lack of the required sensitivity could be a barrier to their use for AFL surveillance, because the LOD/LOQ should be as low as technically possible for accurate dietary exposure assessments. This is due to the fact that

many foods that might be expected to contain AFL do not contain *detectable* AFL contamination, and the default value assigned to those censored samples will affect the estimated dietary exposures (upper-bound estimates only).

3. SAMPLING PROTOCOLS

Almost all of the submitted data on AFL were derived using sampling plans designed for regulatory purposes (Institute of Standards and Industrial Research of Iran, 2000; Turkish Ministry of Agriculture and Rural Affairs and Ministry of Health, 2002, 2003; European Commission, 2006; Japanese Office of Imported Food Safety, 2006; Almond Board of California, 2007; United States Food and Drug Administration, 2007a, 2007b). The producing countries that submitted data to the Committee presented sampling plans similar to or the same as those following EC No. 401/2006 (European Commission, 2006) for the determination of AFL, which includes edible nuts and dried figs. It is not clear, however, how the sampling plan from the EC was derived, nor has the EU published the operating characteristic curves associated with this plan for the various commodities. Thus, producers and importers do not know the "producer risk" associated with operating this plan, nor it is clear to those concerned with food safety what the "consumer risks" are. It is probable that these sampling plans, which cover a range of commodities, such as dried figs, groundnuts and other nuts of very different sizes and for which in many instances little is known about AFL distribution, have all been derived by extrapolation from work on AFL distributions in lots of peanuts (Whitaker et al., 1995; Gilbert & Anklam, 2002), the most studied commodity. Adams & Whitaker (2004) derived the operating characteristic curves that predict the risk of misclassifying a lot associated with the EU sampling plans for raw shelled peanuts and ready-to-eat peanuts. Whitaker (2007) has also derived the operating characteristic curves for the EU plan for almonds, hazelnuts and pistachios.

It was observed that in some producing countries, there are two sampling plans: one for commodity to be exported to the EU, and the second for commodity to be exported to other countries with less strict regulations (Ministério da Agricultura, Pecuária e Abastecimento, 2002, 2004; Almond Board of California, 2007; United States Food and Drug Administration, 2007a, 2007b). There remains a need for harmonized sampling plans, both between different countries and within the same country.

For three of the products to be considered at the present meeting, the uncertainty evaluation was included in the Codex discussion paper CX/CF 07/1/9 on maximum levels of AFT in ready-to-eat almonds, hazelnuts and pistachios (Codex Committee on Contaminants in Food, 2007). It is interesting to point out that the uncertainty was not the same for the three analysed tree nuts (Codex Committee on Contaminants in Food, 2007: p. 46), and this result reinforces the need to evaluate the uncertainty in each particular product. It was proposed (Whitaker, 2006; Whitaker et al., 2006; Codex Committee on Contaminants in Food, 2007) that the performance of sampling plan designs for Brazil nuts be predicted using the distribution and uncertainty equations for almonds and adjusted using the count per unit mass for Brazil nuts. However, the counts per unit mass for shelled almonds,

hazelnuts and pistachios are 773, 1000 and 1600 kernels per kilogram, respectively, whereas those for Brazil nuts and dried figs are in the order of 220 and 45 per kilogram, respectively (Steiner et al., 1988; Whitaker, 2006; Whitaker et al., 2006). Besides the differences in the counts per unit mass, the type of contamination (i.e. the different relationship between AFB_1 and AFT found in almonds and Brazil nuts in European Food Safety Authority [2007: p. 27] and in the submitted data on dried figs) could also contribute to different uncertainties.

To evaluate the total uncertainty among sample test results, it is recommended that the procedure described by Ozay et al. (2006) and Whitaker et al. (2006) or a similar procedure be followed.

Dr Tom Whitaker (United States Department of Agriculture) and Dr Eugenia Vargas (Brazil Ministry of Agriculture) are evaluating the total variability associated with sampling, sample preparation and analytical test procedures for AFL in Brazil nuts, which will provide a base for statistically measuring the effectiveness of sampling plans in this nut. In connection with dried figs, only the contamination distribution is available, and research should be conducted to evaluate uncertainty in sample preparation and analytical test procedures.

The Committee noted that AFL sampling plans should be determined by data relating to contamination distributions and uncertainties within the particular foodstuff. The resulting knowledge of the uncertainty among sample test results should allow each country to refine its sampling plans using, for example, larger sample sizes and/or fewer analytical repetitions in order to meet harmonized criteria. The Committee noted that the data received for this analysis were robust.

The fact that the performance of a sampling plan is in part a function of the lot concentration at which the sampling plan is applied has led to a method by which the plan performance can be predicted by linking it to the AFL lot-to-lot distribution in the crop or the foodstuffs. With the submitted data, to analyse the lot-to-lot distribution of AFT and AFB_1 in relation to Brazil nuts, the log-transformed data on AFT only for values greater than the LOQ for the 2005 and 2006 Brazil nut productions were considered. The results suggest that to provide an adequate model for the lot-to-lot distribution, more data obtained using the same sampling methodology are needed.

It was not possible to identity the lot-to-lot AFL distribution for dried figs because of the small number of positive samples. Only 12.1% of the 53 692 (Turkey, Germany and the USA) analyses submitted tested positive.

There are only a few reports on AFL occurrence in processed products (Abdulkadar et al., 2002, 2004; Aycicek et al., 2005; Chun et al., 2007; Var et al., 2007), and there is a lack of distribution data on them. To improve sampling plans, it is necessary to consider the differences among product types (Samar et al., 2003; MacArthur et al., 2006). Foods are totally different in terms of mycotoxin distribution owing to the mixing effects during processing. There are processed products for which the raw material is ground, such as almonds, figs or hazelnut paste, and others for which it is not, such as hulva. The occurrence and distribution of AFL

contamination should be more homogeneous (probably a normal distribution curve) among packages in lots of pastes, for example, than in hulva lots.

4. EFFECTS OF FOOD PROCESSING

Although AFL are highly stable, studies have indicated that they are degraded in contaminated food by heat treatment. The extent of AFL degradation achieved by roasting was analysed in different substrates, and the results showed that the extent of the reduction depends on the initial level of contamination, heating temperature, moisture content and heating duration (Rustom, 1997). Yazdanpanah et al. (2005) studied the effect of roasting on AFL in contaminated pistachio nuts. Roasting of pistachio nuts in two different ways, salted and unsalted, has traditionally been used to preserve and increase their shelf life. For example, the roasting of pistachio nuts at 150 ºC for 30 min reduced AFL levels by 63% when the initial level was 44 µg AFB_1/kg, 24% when the initial level was 213 µg AFB_1/kg, 17% when the initial level was 21.9 µg AFB_1/kg and 47% when the initial level was 18.5 µg AFB_2/kg.

Among the submitted data on roasteries from the United Arab Emirates, a few samples in the last 6 months of 2006 were from pistachios, both roasted and unroasted. The roasted samples presented an average contamination of 7.6 µg/kg, with a maximum of 70 µg/kg (only one positive); the unroasted samples presented an average level of 127 µg/kg (only one with a non-detected level), with a maximum of 430 µg/kg.

Yazdanpanah et al. (2005) suggested that co-administration of some commonly used food additives with roasting may accelerate the destruction of AFL in pistachios even under more gentle roasting conditions. It was previously reported that boiling raw unshelled peanuts with 5% sodium chloride water solution can reduce AFL up to 80% (Farah et al., 1983). It has been suggested that the presence of water helps in opening the lactone ring in AFB_1 (by the addition of a water molecule to the ring) to form a terminal carboxylic acid. The terminal acid group thereafter undergoes heat-induced decarboxylation (Rustom, 1997). The presence of ionic salts will probably increase the extent of AFL degradation by heat in salted roasted nuts.

5. LEVELS AND PATTERNS OF CONTAMINATION OF FOOD COMMODITIES

AFL concentration data for different food items, in particular tree nuts (edible portion) such as almonds, Brazil nuts, hazelnuts and pistachios, from 2001 to 2006 were evaluated for the current meeting from several producing and importing countries and regions (Australia, Brazil, EU, Islamic Republic of Iran, Japan, Turkey, United Arab Emirates and USA). The starting point for the compilation of the JECFA database from all data submitted by countries at this meeting was the EFSA EU monitoring database for AFL levels in food, compiled for the EFSA risk assessment requested by the EC in 2006 (European Food Safety Authority, 2007).

It was assumed that when information was missing from submitted data, the LOD for AFT was twice the LOD of AFB_1 and the LOQ was 3.3 times the LOD, as assumed in the European Food Safety Authority (2007) risk assessment.

Owing to the relatively low LOD obtained by Member States as reported in submitted data, the high number of data points reported below the LOD or LOQ for the four nuts included in this assessment (>60%) and the need to have understandable tables and figures, the upper-bound AFL concentration level was used in the dietary exposure estimates for reporting purposes (GEMS/Food-Euro, 1995).

5.1 National surveillance data

5.1.1 Australia

Australia submitted a report from the Department of Health (Government of Western Australia, 2005), with occurrence data for 109 individual samples of different nut samples prepared as ready-to-eat, for almond (19), Brazil nut (3), cashew (9), chestnut (4), hazelnut (15), macadamia (22), pecan (3), pine (3), pistachio (15) and walnut (16), based on food surveys of AFL conducted in 2003 and 2004. Sixteen per cent of these samples had quantified levels of AFL. AFL levels ranged between not detected (<2 µg/kg) and 11 µg/kg, with no level in excess of the 15 µg/kg limit in Australia.

5.1.2 Brazil

Brazil submitted occurrence data for 329 individual results of Brazil nut lots to be exported to the EU (35 lots) and other countries (294 lots). The data refer to in-shell (processed and unprocessed) and shelled Brazil nuts, and in all cases only the edible portion (kernels) was analysed. AFL concentrations were reported as below the LOD for 85% of the exported nut lots. The most common LOD reported was 0.5–0.6 µg/kg for AFB_1 and 0.3–0.5 µg/kg for other AFL (AFB_2, AFG_1 and AFG_2). Average concentration levels from Brazil nut lots were around 8.5 µg/kg for AFB_1 and 20 µg/kg for AFT (as shown in Table 4 below). The legal regulatory limit in Brazil is 30 µg/kg for AFT for Brazil nut lots sold in the Brazilian market.

5.1.3 European Union

A total of 49 748 analytical results for AFL were submitted from 22 EU Member States from 2000 to 2006 in response to a call for information issued by the EC.

After some data were discarded, as described in the EFSA scientific opinion (European Food Safety Authority, 2007), 34 326 analytical results were included in the EFSA analysis, submitted by Austria (1453), Belgium (434), Cyprus (212), Czech Republic (1464), Denmark (340), Estonia (349), Finland (1419), France (2719), Germany (5287), Greece (4847), Hungary (3750), Ireland (1765), Italy (6959), Latvia (549), Luxembourg (320), Slovakia (939), Slovenia (402), Spain (229), Sweden (211) and the United Kingdom (678). Most of these results came

from the official food control authorities in respective Member States and comprised both random and targeted sampling, thus introducing both uncertainty and variability. These analytical results comprised data for almonds (1768), Brazil nuts (622), hazelnuts (3163), pistachios (4069), figs (2067), other dried fruits (1472), other nuts (peanut, cashew, walnut, coconut) (9943), maize (961), cocoa products (248), oil of groundnut (peanut butter) (496), oil seeds (339), rice (541), other cereals (wheat, barley, oat, rye) (2304), spices (4704) and other foodstuffs (1028).

AFL concentration levels were reported as below the LOD for 25 451 of the European samples, whereas levels above the LOD were found in 8875 or 26% of the samples. The most common LOD reported was 0.1 or 0.2 µg/kg for AFB_1 and 0.2 or 0.4 µg/kg for AFT after some adjustment for missing values in relation to the LOD in some samples. The distribution of AFL contamination in foods in the EU is already described in the EFSA opinion (European Food Safety Authority, 2007).

5.1.4 Islamic Republic of Iran

The Islamic Republic of Iran submitted the results of 6187 AFL monitoring data for 1849 pistachio nut lots (ready-to-eat) consigned to be exported from July 2004 to March 2007 (Secretariat of Iran Codex Committee on Contaminants in Food, 2007). The sensitivity of the analytical method was reported with an LOD of 0.2 µg/kg for AFB_1 and 0.4 µg/kg for AFT, with 24% of the data reported below the LOD. A linear regression coefficient of 1.13 (similar to the number reported in the EFSA opinion) was applied to estimate the level of AFT in a low number of samples (around 4%) in which AFB_1 data only were submitted.

5.1.5 Japan

Using the Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme (GEMS/Food) reporting format, Japan submitted aggregated monitoring results of inspection control for AFB_1 for pistachios (2342), walnuts (3427) and almonds (6706) and individual data for AFB_1 from a retail food survey for pistachios (159), almonds (103), walnuts (23) and hazelnuts (13). Sampling was performed during the 2000–2003 period for inspection control purposes and during the 1988–2006 period for the retail food survey (Sugita-Konishi et al., 2007). Both the LOD and LOQ ranged between 0.05 and 0.2 µg/kg for AFB_1 and between 0.1 and 1 µg/kg for AFT.

Results from monitoring data for AFB_1 for hazelnuts, almonds and pistachios were reported as 29.4, 9.4 and 7.4 µg/kg, respectively, with less than 3% of values below the reporting limits (LOD and LOQ), assumed to be zero for a lower-bound estimate. For the retail food survey, average AFB_1 and AFT levels for pistachios were reported to be 5.8 and 6.1 µg/kg, respectively, and for almonds, 0.02 and 0.03 µg/kg, respectively; the reporting limit values (LOD and LOQ) were assumed to be zero for a lower-bound estimate, where 96% of the results for pistachios and 92% of the results for almonds were less than the LOD or LOQ.

The present regulatory limit in Japan is 10 µg/kg for AFB_1 in all foods.

5.1.6 Turkey

Turkey submitted occurrence data for 37 622 samples of dried figs to be exported for the 2003–2006 period (Turkish Ministry of Agriculture and Rural Affairs, 2007). AFL concentrations were reported as below the reporting limit (LOD and LOQ) for 8% of the dried fig samples. The most common LOD reported for AFB_1 was 0.2–0.3 µg/kg, and the LOD ranged between 0.1 and 0.4 µg/kg for the other AFL (AFB_2, AFG_1 and AFG_2). Average AFL concentrations for Turkish exported dried figs were around 0.6 µg/kg for AFB_1 and 1.0 µg/kg for AFT (as shown in Table 5 below).

5.1.7 United Arab Emirates

AFL data submitted by the United Arab Emirates were the results of AFL tests (AFB_1, AFB_2, AFG_1 and AFG_2) in food and feed for 2005 conducted in the Food and Environment Laboratory of Dubai Municipality. All samples were from retailers (roasteries, general markets and hypermarkets). Ninety-nine per cent of imported samples of nuts available in the Emirate of Abu Dhabi in 2005 were from both the Dubai and Sharjah ports. The method of analysis was per SOP-FE-2320 using immunoaffinity column cleanup with HPLC-FLD based on AOAC International official methods (AOAC International, 2000a). The sensitivity of the analytical method was reported with an LOD of 0.7 µg/kg for AFB_1 and 1.0 µg/kg for AFT. A total of 591 individual results for AFB_1 and AFT were provided for other nuts (groundnuts) (62%), pistachios (17%), sweet products containing nuts (19%), butter of Karité nut (5%), cocoa products (3%) and dried fruits (<1%). Forty per cent of those data were reported below the LODs. Average concentrations in AFB_1 and AFT were reported at the upper-bound level to be 13.0 and 17.5 µg/kg for other nuts, 49.1 and 53.4 µg/kg for pistachios, 11.6 and 12.8 µg/kg for sweet products, 8.3 and 10 µg/kg for butter of Karité nut, 3.7 and 4.7 µg/kg for cocoa products and 8.9 and 10.4 µg/kg for dried figs, respectively.

5.1.8 United States of America

AFL levels in domestic and imported samples of tree nuts (almonds, Brazil nuts, hazelnuts and pistachios) and dried figs were provided at this meeting by the United States Food and Drug Administration (2006). Samples were collected during routine inspections of firms that stored and/or distributed domestic and imported foods during the 1996–2006 period. In total, 1310 results for tree nuts—almonds (44%, with more than 80% from domestic), Brazil nuts (15%), hazelnuts (16%) and pistachios (25%, with more than 65% from domestic)—and 103 for dried figs from domestic and imported samples were submitted. The LOQ for AFT was about 1 µg/kg, with less than 10% of data reported to be below this limit. The present regulatory limit in the USA for AFT in tree nuts is 20 µg/kg.

5.2 International surveillance data from countries importing tree nuts and dried figs

The Committee decided at this meeting to base the assessment of the impact of different MLs for AFT of 4, 8, 10, 15 and 20 µg/kg on dietary exposure estimates on data submitted from countries that were producers of almonds, Brazil nuts, hazelnuts, pistachios and dried figs only, to best represent AFL contamination levels of these products prior to import restrictions.

5.2.1 *Almonds*

The main almond producers in the world are the USA (42%), Spain (13%), the Syrian Arab Republic (8%), Italy (7%) and the Islamic Republic of Iran (5%) (FAOSTAT, 2007). The statistical distribution of AFB_1 and AFT levels (mean, coefficient of variation [CV] and percentiles) for almonds from producers is given in Table 1, as well as the impact of different MLs for AFT (4, 8, 10, 15 and 20 µg/kg) on this distribution and the corresponding proportion of rejected samples from the world market for each scenario. Data were also submitted from the USA (568), EU (1766) and Japan (56) as importing countries. Mean concentration levels for AFT were not significantly different between the USA and other countries (1.6 vs 2.0 µg/kg, $P < 0.001$), where 82% of data were reported to be below the reporting limits (LOD and LOQ).

Data in Table 1 show that the actual mean concentration of AFT in almonds from the main export country markets is around 2 µg/kg. Setting MLs for AFT from 20 to 4 µg/kg should result in mean concentrations of AFT approximately 2–3 times lower than the actual mean concentration of AFT (from 0.8 to 0.6 µg/kg vs 2 µg/kg). The proportion of rejected almond samples from the world market would be between 1% for an ML set at 20 µg/kg and 3% for an ML set at 4 µg/kg.

5.2.2 *Brazil nuts*

The main producing region in the world is South America (Brazil, Bolivia, Ecuador and Peru) (International Tree Nut Council, 2002). The statistical distribution of AFB_1 and AFT levels (mean, CV and percentiles) for Brazil nuts from producers is given in Table 2, as well as the impact of different MLs for AFT (4, 8, 10, 15 and 20 µg/kg) on this distribution and the corresponding proportion of rejected samples from the world market for each scenario. Data were submitted from Brazil (329) for the 2005–2006 period, where AFL concentrations were reported as below the LOD for 85% of the exported nut lots.

Data in Table 2 show that the actual mean concentration of AFT in Brazil nut lots for export is around 20 µg/kg. Setting MLs for AFT going from 20 to 4 µg/kg should result in mean concentrations of AFT approximately 10 times lower than the actual mean concentration of AFT (from 2.4 to 1.7 µg/kg vs 20 µg/kg). The proportion of rejected Brazil nut lots from the world market would be between 11% for an ML set at 20 µg/kg and 17% for an ML set at 4 µg/kg.

Table 1. Summary of the impact of different proposed MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT content (upper-bound scenario) in almonds for 2000–2007, including the predicted proportion of rejected samples from the world market

Scenario	Type	No. of samples	Mean (µg/kg)[a]	CV (%)[b]	AFL content (µg/kg)									% of rejected samples
					P5	P25	P50	P60	P75	P90	P95	P97.5	Max.	
All data	AFB_1	2390	1.3	1189	0.1	0.1	0.2	0.2	0.5	0.5	1.5	4.1	575	0
	AFT		2.0	940	0.1	0.2	0.4	0.4	1.0	1.0	2.5	6.2	579	
ML 20 µg/kg	AFB_1	2361	0.5	260	0.1	0.1	0.2	0.2	0.5	0.5	1.2	2.1	16	1
	AFT		0.8	206	0.1	0.2	0.4	0.4	1.0	1.0	1.8	3.3	18	
ML 15 µg/kg	AFB_1	2354	0.4	234	0.1	0.1	0.2	0.2	0.5	0.5	1.1	2.0	13	2
	AFT		0.7	185	0.1	0.2	0.4	0.4	1.0	1.0	1.6	3.0	15	
ML 10 µg/kg	AFB_1	2337	0.4	188	0.1	0.1	0.2	0.2	0.5	0.5	1.0	1.5	10	2
	AFT		0.7	143	0.1	0.2	0.4	0.4	1.0	1.0	1.4	2.4	10	
ML 8 µg/kg	AFB_1	2325	0.3	152	0.1	0.1	0.2	0.2	0.5	0.5	0.9	1.3	6	3
	AFT		0.6	119	0.1	0.2	0.4	0.4	1.0	1.0	1.3	2.1	8	
ML 4 µg/kg	AFB_1	2307	0.3	121	0.1	0.1	0.2	0.2	0.5	0.5	0.8	1.2	4	3
	AFT		0.6	97	0.1	0.2	0.4	0.4	1.0	1.0	1.2	1.7	5	

Max., maximum; Px, xth percentile.

[a] Concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.

[b] Coefficient of variation (standard deviation divided by mean, %).

Table 2. Summary of the impact of different proposed MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT content (upper-bound scenario) in Brazil nuts for 2005–2006, including the predicted proportion of rejected samples from the world market

Scenario	Type	No. of samples	Mean (µg/kg)[a]	CV (%)[b]	AFL content (µg/kg)									% of rejected samples
					P5	P25	P50	P60	P75	P90	P95	P97.5	Max.	
All data	AFB_1	329	8.5	406	0.50	0.5	0.5	0.5	0.6	14.9	44.7	71.6	425	0
	AFT		19.8	399	1.60	1.6	1.6	1.6	1.7	21.9	114.9	161.1	873	
ML 20 µg/kg	AFB_1	292	1.0	205	0.50	0.5	0.5	0.5	0.5	1.3	4.0	6.1	18	11
	AFT		2.4	119	1.60	1.6	1.6	1.6	1.6	3.0	6.9	12.1	19	
ML 15 µg/kg	AFB_1	286	0.8	158	0.50	0.5	0.5	0.5	0.5	1.0	2.0	4.0	12	13
	AFT		2.1	87	1.60	1.6	1.6	1.6	1.6	2.5	3.9	6.9	15	
ML 10 µg/kg	AFB_1	281	0.7	47	0.50	0.5	0.5	0.5	0.5	0.6	1.6	3.2	5	15
	AFT		1.9	27	1.60	1.6	1.6	1.6	1.6	1.7	3.1	4.2	9	
ML 8 µg/kg	AFB_1	277	0.6	77	0.50	0.5	0.5	0.5	0.5	0.6	1.3	1.7	4	16
	AFT		1.8	32	1.60	1.6	1.6	1.6	1.6	1.7	3.0	3.5	6	
ML 4 µg/kg	AFB_1	272	0.6	43	0.50	0.5	0.5	0.5	0.5	0.6	1.0	1.4	2	17
	AFT		1.7	21	1.60	1.6	1.6	1.6	1.6	1.7	2.5	3.0	4	

Max., maximum; Px, xth percentile.

[a] Concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.

[b] Coefficient of variation (standard deviation divided by mean, %).

5.2.3 Hazelnuts

Turkey is the primary producing country for hazelnuts, covering approximately 70% of the world market (FAOSTAT, 2007), but the Committee received no data on AFT levels in hazelnuts from Turkey; therefore, the Committee chose to use all of the submitted data supplied by the EU (3163), USA (215) and Japan (6) for its analyses on the distribution statistics for AFB_1 and AFT concentrations. Seventy-seven per cent of the data were reported to be below the reporting limits (LOD and LOQ).

The statistical distribution of AFB_1 and AFT levels (mean, CV and percentiles) for hazelnuts is given in Table 3, as well as the impact of different MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on this distribution and the corresponding proportion of rejected samples from the world market for each scenario. The actual mean concentration of AFT in hazelnuts from the main importing country markets was around 2 µg/kg. Setting MLs for AFT for hazelnuts from 20 to 4 µg/kg should result in mean concentrations approximately 2–4 times lower than the actual mean concentration of AFT in hazelnuts (from 1 to 0.6 µg/kg vs 2 µg/kg). The proportion of rejected hazelnut samples from the world market would be between 1% for an ML set at 20 µg/kg and 7% for an ML set at 4 µg/kg.

5.2.4 Pistachios

The main country producer for which data were submitted was the Islamic Republic of Iran. This country accounts for around 65% of the world's export market for pistachios (FAOSTAT, 2007).

The statistical distribution of AFB_1 and AFT levels (mean, CV and percentiles) for pistachios from the Islamic Republic of Iran is given in Table 4, as well as the impact of different MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on this distribution and the corresponding proportion of rejected samples from the world market for each scenario. The actual mean concentration of AFB_1 or AFT in pistachios is around 50 µg/kg. Setting MLs for AFT from 20 to 4 µg/kg should result in mean concentrations approximately 10–50 times lower than the actual mean concentration (from 4 to 1 µg/kg vs 50 µg/kg). The proportion of rejected pistachio samples from the world market would be between 40% for an ML set at 20 µg/kg and 60% for an ML set at 4 µg/kg.

Table 3. Summary of the impact of different proposed MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT content (upper-bound scenario) in hazelnuts for 1996–2007, including the predicted proportion of rejected samples from the world market

Scenario	Type	No. of samples	Mean (µg/kg)[a]	CV (%)[b]	AFL content (µg/kg)									% of rejected samples
					P5	P25	P50	P60	P75	P90	P95	P97.5	Max.	
All data	AFB_1	3383	1.0	783	0.04	0.1	0.2	0.2	0.5	1.3	2.8	4.5	334	0
	AFT		1.9	714	0.08	0.2	0.4	0.4	1.0	2.7	6.0	9.9	630	
ML 20 µg/kg	AFB_1	3340	0.5	241	0.04	0.1	0.2	0.2	0.5	1.1	2.3	3.4	17	1
	AFT		1.0	222	0.08	0.2	0.4	0.4	1.0	2.2	4.4	7.5	20	
ML 15 µg/kg	AFB_1	3317	0.5	217	0.04	0.1	0.2	0.2	0.4	1.0	2.1	3.0	14	2
	AFT		0.9	198	0.08	0.2	0.4	0.4	0.9	2.1	3.9	6.1	15	
ML 10 µg/kg	AFB_1	3285	0.4	194	0.04	0.1	0.2	0.2	0.4	1.0	1.8	2.9	9	3
	AFT		0.8	178	0.08	0.2	0.4	0.4	0.8	1.9	3.6	4.9	10	
ML 8 µg/kg	AFB_1	3246	0.4	171	0.04	0.1	0.2	0.2	0.4	0.8	1.6	2.1	7	4
	AFT		0.7	156	0.08	0.2	0.3	0.4	0.7	1.7	3.1	3.8	8	
ML 4 µg/kg	AFB_1	3161	0.3	142	0.04	0.1	0.2	0.2	0.3	0.6	1.1	1.6	4	7
	AFT		0.6	126	0.08	0.2	0.3	0.4	0.6	1.2	2.2	3.0	4	

Max., maximum; Px, xth percentile.

[a] Concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.

[b] Coefficient of variation (standard deviation divided by mean, %).

Table 4. Summary of the impact of different proposed MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT content (upper-bound scenario) for pistachios from the Islamic Republic of Iran for 2004–2007, including the predicted proportion of rejected samples from the world market

Scenario	Type	No. of samples	Mean (µg/kg)[a]	CV (%)[b]	AFL content (µg/kg)									% of rejected samples
					P5	P25	P50	P60	P75	P90	P95	P97.5	Max.	
All data	AFB_1	1849	49.2	216	0.2	1.2	8.6	17.8	46.8	133.6	243.7	331.0	1411.0	0
	AFT		54.4	212	0.4	1.2	9.6	19.7	51.2	150.9	269.5	368.1	1418.8	
ML 20 µg/kg	AFB_1	1112	3.95	120	0.2	0.2	2.0	2.9	5.9	11.7	14.7	16.4	19.2	40
	AFT		4.35	118	0.4	0.4	2.0	3.1	6.5	12.8	16.0	17.4	19.9	
ML 15 µg/kg	AFB_1	1036	3.07	116	0.2	0.2	1.6	2.5	4.7	8.9	10.6	12.0	14.3	44
	AFT		3.40	113	0.4	0.4	1.7	2.6	5.0	9.8	12.1	13.2	15.0	
ML 10 µg/kg	AFB_1	935	2.20	111	0.2	0.2	1.2	2.0	3.4	6.2	7.7	8.3	9.4	49
	AFT		2.43	106	0.4	0.4	1.3	2.1	3.6	6.8	8.4	9.0	9.9	
ML 8 µg/kg	AFB_1	878	1.81	107	0.2	0.2	1.0	1.7	2.8	4.8	5.9	6.5	7.7	53
	AFT		2.01	101	0.4	0.4	1.0	1.8	3.0	5.3	6.5	7.1	8.0	
ML 4 µg/kg	AFB_1	722	1.06	100	0.2	0.2	0.5	0.9	1.8	2.8	3.2	3.4	4.0	61
	AFT		1.20	86	0.4	0.4	0.6	1.0	1.9	2.9	3.4	3.6	4.0	

Max., maximum; Px, xth percentile.

[a] Concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.

[b] Coefficient of variation (standard deviation divided by mean, %).

5.2.5 *Dried figs*

Turkey is the main country producing dried fruits, covering approximately 63% of the world market (Seker, 2007). The statistical distribution of AFB_1 and AFT levels (mean, CV and percentiles) for dried fruits from producers is given in Table 5, as well as the impact of different MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on this distribution and the corresponding proportion of rejected samples from the world market for each scenario. A large number of data (40 822 individual data) were provided for dried figs at this meeting by Turkey for the 2003–2006 period.

Data in Table 5 show that the actual mean concentration of AFT in dried figs from the main export country market is around 1.0 µg/kg. Setting MLs for AFT going from 20 to 4 µg/kg should result in mean concentrations approximately 2 times lower than the actual mean concentration of AFT (from 0.6 to 0.4 µg/kg vs 1.0 µg/kg). The proportion of rejected dried fruit samples from the world market would be between 1% for an ML set at 20 µg/kg and 3% for an ML set at 4 µg/kg.

5.3 *Summary of aflatoxin occurrence and levels in food commodities and the potential effect of MLs in almonds, Brazil nuts, hazelnuts, pistachios and dried figs*

AFL occurrence data on almonds, Brazil nuts, hazelnuts, pistachios and dried figs were obtained from both producing and importing countries. The Committee decided to base the assessment of the impact of different MLs (4, 8, 10, 15 and 20 µg/kg) for AFT for almonds, Brazil nuts, hazelnuts, pistachios and dried figs on data provided by producing countries, as these are more likely to represent the actual occurrence of AFL in the commodities. The primary producing countries or regions (FAOSTAT, 2007) were, for almonds, the USA (42% of the world market); for Brazil nuts, South America (100%); for hazelnuts, Turkey (70%); for pistachios, the Islamic Republic of Iran (65%); and for dried figs, Turkey (63% for dried fruits). Turkey is the primary producing country for hazelnuts, but the Committee received no data on AFT levels in hazelnuts from Turkey; therefore, the Committee chose to use all of the submitted data supplied by the EU, USA and Japan for its analyses.

The mean concentrations of AFT in nuts and dried figs in the main producing countries were, for almonds, 2 µg/kg; for Brazil nuts, 20 µg/kg; for hazelnuts, 2 µg/kg; for pistachios, 54 µg/kg; and for dried figs, 1 µg/kg. The effects of the theoretical full enforcement of MLs (all samples above the ML would be excluded from the distribution) at 20, 15, 10, 8 and 4 µg/kg are shown in Table 6. The reductions in mean AFT concentrations would be approximately 2- to 3-fold for almonds, 10-fold for Brazil nuts, 2- to 4-fold for hazelnuts, 10- to 50-fold for pistachios and 2-fold for dried figs. The corresponding proportion of rejected samples would be 1–3% for almonds, 11–17% for Brazil nuts, 1–7% for hazelnuts, 40–60% for pistachios and 1–3% for dried figs.

Table 5. Summary of the impact of different proposed MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT content (upper-bound scenario) in dried figs for 2003–2006, including the predicted proportion of rejected samples from the world market

Scenario	Type	No. of samples	Mean (µg/kg)[a]	CV (%)[b]	AFL content (µg/kg)									% of rejected samples
					P5	P25	P50	P60	P75	P90	P95	P97.5	Max.	
All data	AFB_1	40 822	0.6	821	0.06	0.20	0.20	0.20	0.20	0.30	1.02	2.21	424	0
	AFT		1.0	689	0.07	0.40	0.40	0.40	0.40	0.56	1.63	3.40	424	
ML 20 µg/kg	AFB_1	40 537	0.4	272	0.06	0.20	0.20	0.20	0.20	0.28	0.83	1.67	20	1
	AFT		0.6	230	0.07	0.40	0.40	0.40	0.40	0.56	1.27	2.59	20	
ML 15 µg/kg	AFB_1	40 431	0.3	230	0.06	0.20	0.20	0.20	0.20	0.28	0.77	1.54	15	1
	AFT		0.6	194	0.07	0.40	0.40	0.40	0.40	0.56	1.18	2.35	15	
ML 10 µg/kg	AFB_1	40 274	0.3	1031	0.06	0.20	0.20	0.20	0.20	0.28	0.69	1.60	10	1
	AFT		0.5	852	0.07	0.40	0.40	0.40	0.40	0.56	1.06	2.05	10	
ML 8 µg/kg	AFB_1	40 167	0.3	168	0.06	0.20	0.20	0.20	0.20	0.28	0.64	1.20	8	2
	AFT		0.5	136	0.07	0.40	0.40	0.40	0.40	0.56	0.98	1.88	8	
ML 4 µg/kg	AFB_1	39 731	0.3	113	0.06	0.20	0.20	0.20	0.20	0.28	0.51	0.85	5	3
	AFT		0.4	89	0.07	0.20	0.40	0.40	0.40	0.56	0.75	1.30	4	

Max., maximum; Px, xth percentile.

[a] Concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.

[b] Coefficient of variation (standard deviation divided by mean, %).

Table 6. Impact of different hypothetical ML scenarios for AFT on the mean AFT level and the corresponding proportion of rejected samples from the producing countries on the world market for tree nuts and dried figs

Scenario	Mean AFT level, µg/kg (proportion of rejected samples, %)					
	No MLs	ML 20 µg/kg	ML 15 µg/kg	ML 10 µg/kg	ML 8 µg/kg	ML 4 µg/kg
Almonds	2.0 (0)	0.8 (1)	0.7 (2)	0.7 (2)	0.6 (3)	0.6 (3)
Brazil nuts	20 (0)	2.4 (11)	2.1 (13)	1.9 (15)	1.8 (16)	1.7 (17)
Hazelnuts	1.9 (0)	1.0 (1)	0.9 (2)	0.8 (3)	0.7 (4)	0.6 (7)
Pistachios	54 (0)	4.4 (40)	3.4 (44)	2.4 (49)	2.0 (53)	1.2 (61)
Dried figs	1.0 (0)	0.6 (1)	0.6 (1)	0.5 (1)	0.5 (2)	0.4 (3)

5.4 Other foods contributing to total dietary aflatoxin exposure

Food sources other than tree nuts (almonds, Brazil nuts, hazelnuts and pistachios) and dried figs can contribute to the overall dietary exposure to AFT in humans. Occurrence data available from the last JECFA evaluation (Annex 1, reference *132*) and the EFSA opinion on AFL (European Food Safety Authority, 2007) were used with the corresponding amount of foods available for consumption from the GEMS/Food Consumption Cluster Diets to estimate AFT exposures (see section 6). In describing these contributing food sources, the Committee decided to focus mainly on food sources with detectable AFL levels that could be considered as being a contributing food source to average overall dietary exposure. This food prioritization was made by the Committee to avoid overestimating overall exposure to AFL. For example, for some cereals (except maize and rice), there were few detected AFL levels reported in EU data (below 5%) or too few data from only one country region (e.g. Brazil; Annex 1, reference *132*), which could not be extrapolated to other regions.

The majority of the data included in the estimation of dietary AFT exposure from other food sources came from the EU. The Committee noted that the European data do not reflect the actual mean concentration in other world regions for some foods considered here, as the mean concentration of AFT in the EU takes into account fewer highly contaminated samples as a result of existing EU MLs compared with regions with higher MLs or lack of enforcement.

Based on these considerations and submitted data in foods other than tree nuts and dried figs, the food commodities included in the average overall exposure were maize (961), groundnuts (i.e. peanuts, 9132) and other nuts (i.e. walnut, cashew, chestnut, macadamia, pecan, 1177), dried fruits other than figs (apricots, plums, grapes, dates and others, 1477), spices (4704), cocoa and cocoa products (cocoa mass, cocoa butter, cocoa powder, 266), rice (541), oil of groundnut (peanut butter, peanut cream, 496), oilseeds (339) and butter of Karité nut (29). A summary of the distribution of AFB_1 and AFT levels observed in these foods is given in Table 7. Most of the data considered in the dietary exposure assessment came from

the EU and the United Arab Emirates (for all data on butter of Karité nut, around 7% for cocoa products and 4% for other nuts). Because the European data do not reflect the actual mean concentration in other world regions for some foods, the mean upper-bound level has preferentially been used in the dietary exposure estimates in all 13 GEMS/Food Consumption Cluster Diets (see section 6).

This analysis indicates that the mean concentrations of AFB_1 and AFT were less than 1 µg/kg for all foods except spices, cocoa products, groundnuts and butter of Karité nut, for which the mean levels ranged from 2 to 4 µg/kg.

For rice, different concentrations were reported in different regions (producing and non-producing countries). A survey of AFB_1 in rice was conducted on 108 food-grade rice samples randomly collected during July and August 2002 in Seoul, Republic of Korea; the mean level was of the same order of magnitude as that described for EU data (Park et al., 2004). Naturally occurring AFB_1 was found in 5 out of 108 (6%) samples of rice, with a mean level of 4.8 ng/g for samples with detected levels only. The LOD gave a mean upper bound of around 1.2 µg/kg in rice marketed in Seoul. JECFA also reported an average concentration of around 2 µg/kg in rice from Brazil, with 10% of data below the LOD (Annex 1, reference *132*). The EU database on AFL reported a mean AFT level around 0.6–1.0 µg/kg in the EU (see Table 7). Another survey from Qatar reported AFT levels around 0.1–0.2 µg/kg (Abdulkadar et al., 2004), and other countries, including Japan and Argentina, reported no detected levels (Broggi et al., 1999a, 1999b; Sugita-Konishi et al., 2006). High AFT levels, such as those for peanuts or maize, have never been reported in rice; the highest reliably reported levels are less than 10 µg/kg. Because of these uncertainties in the data, rice was not included in estimating overall dietary exposures to AFT for comparison with the contribution from almonds, Brazil nuts, hazelnuts, pistachios and dried figs. In regions where rice is a major component of the diet, any low levels of AFT in rice may lead to its being a major contributor to total dietary exposure to AFT, even though that exposure may be low when compared with that in other regions.

For maize, a publication review on AFT occurrence data by Williams et al. (2004) reported concentrations 10 times higher than those described in Table 7 (around 33 µg/kg on average in Bangladesh). JECFA also reported a mean level of 35 µg/kg in maize from Brazil, with 51% of data above the LOD. AFB_1 in barley-based food was also reported at an average level of 4.1 µg/kg in the Republic of Korea (Park et al., 2004).

For peanut oil, mean AFT levels were reported to be 40 µg/kg in Senegal (Williams et al., 2004).

For groundnuts, AFB_1 and AFT concentrations reported here are about 4 times lower than concentrations reported by JECFA previously (Annex 1, reference *132*): respectively 2.4 and 3.3 µg/kg vs 7–8.3 and 13–14 µg/kg.

Table 7. Summary of the statistical distributions of AFB_1 and AFT contents in foods other than tree nuts and figs contributing to the total dietary exposure for 2000–2006

Commodity	Type	No. of samples	$n < LOR^a$ (%)	Mean (µg/kg)		CV (%)[d]	AFL content[e] (µg/kg)								
				lb[b]	ub[c]		P5	P25	P50	P60	P75	P90	P95	P97.5	Max.
Maize	AFB_1	961	86	0.1	0.3	194	0.04	0.10	0.14	0.20	0.20	0.50	0.70	1.02	8
	AFT			0.2	0.4	149	0.08	0.20	0.24	0.40	0.40	0.50	1.00	1.54	9
Rice	AFB_1	541	86	0.5	0.8	391	0.04	0.10	0.20	0.50	0.80	1.00	2.00	3.00	57
	AFT			0.6	1.0	366	0.08	0.20	0.40	0.50	0.80	1.00	2.00	4.57	60
Cocoa products	AFB_1	266	68	1.3	1.9	587	0.04	0.10	0.20	0.35	0.50	0.76	1.86	7.31	120
	AFT			1.4	2.1	557	0.08	0.20	0.40	0.50	0.50	1.40	1.88	7.77	138
Oilseeds	AFB_1	339	89	0.3	0.4	441	0.02	0.10	0.10	0.10	0.20	0.23	0.91	1.29	22
	AFT			0.4	0.6	396	0.03	0.20	0.20	0.20	0.40	0.46	1.02	2.63	25
Dried fruits other than figs	AFB_1	1477	93	0.1	0.3	388	0.03	0.10	0.10	0.20	0.20	0.80	1.00	1.00	33
	AFT			0.2	0.6	481	0.06	0.20	0.30	0.40	0.40	1.00	2.00	2.00	90
Groundnuts	AFB_1	9132	83	2.3	2.4	834	0.03	0.10	0.10	0.20	0.20	1.00	4.64	13.03	935
	AFT			3.0	3.3	719	0.04	0.20	0.20	0.40	0.40	2.00	6.72	21.79	985
Other nuts	AFB_1	1177	93	0.8	1.0	1356	0.04	0.10	0.10	0.10	0.20	0.23	1.00	1.20	385
	AFT			1.1	1.3	1170	0.08	0.20	0.20	0.20	0.40	0.46	1.92	2.00	402
Butter of Karité nut	AFB_1	29	28	0.4	3.7	157	0.50	0.70	1.62	1.87	3.33	8.32	16.0	21.40	25
	AFT			4.3	4.7	147	0.57	1.40	1.86	2.21	4.03	9.81	18.6	24.85	30

Table 7. (contd)

Commodity	Type	No. of samples	$n <$ LOR[a] (%)	Mean (µg/kg)		CV (%)[d]	AFL content[e] (µg/kg)								
				lb[b]	ub[c]		P5	P25	P50	P60	P75	P90	P95	P97.5	Max.
Oil of groundnut	AFB_1	496	62	0.5	0.6	284	0.05	0.10	0.20	0.21	0.50	1.07	2.03	3.22	22
	AFT			0.8	0.9	275	0.08	0.20	0.33	0.40	0.60	1.80	3.14	4.42	30
Spices	AFB_1	4704	60	1.3	1.5	312	0.04	0.10	0.20	0.40	1.00	3.10	6.60	9.30	96
	AFT			1.6	1.9	277	0.08	0.20	0.40	0.60	1.52	4.00	7.80	11.3	96

[a] Limits of reporting (LOD or LOQ).
[b] Lower bound: concentrations of less than the LOD or LOQ have been assumed to be equal to 0 or the LOD.
[c] Upper bound: concentrations of less than the LOD or LOQ have been assumed to be equal to those limits.
[d] Coefficient of variation (standard deviation divided by mean, %).
[e] All the statistical distributions for AFL content in the table are expressed for the upper-bound scenario.

6. ESTIMATED DIETARY EXPOSURE

6.1 National assessments of dietary exposure for aflatoxins

Dietary exposure estimates were reported at this meeting for EU Member States from the EFSA opinion (European Food Safety Authority, 2007), by Japan (Sugita-Konishi et al., 2007) and also from the scientific literature.

6.1.1 European Union

The EFSA opinion assessed the influence of changes to the MLs (4, 8 and 10 µg/kg) for almonds, hazelnuts and pistachios on the overall dietary exposure to AFL. In the EFSA opinion, dietary exposure estimates were based on food consumption data from individual dietary records for nuts from representative Member States and on food consumption data from the GEMS/Food Consumption Cluster Diets database of the World Health Organization (2006) for all other foods. Mean AFT occurrence data used in its calculations were those reported in the EFSA opinion.

Dietary AFT exposure estimates for adult high consumers (United Kingdom data for vegan and vegetarians included) of almonds, hazelnuts and pistachios (95th percentile) were derived from a range of individual consumption surveys for four Member States (France, Germany, Spain and the United Kingdom) and mean occurrence data. Dietary exposure estimates ranged between 0.93 and 2.45 ng/kg body weight (bw) per day for the lower- to upper-bound estimates. Almonds, hazelnuts and pistachios contributed between 16% and 22% of the overall dietary exposure to AFL, equivalent to 0.16–0.55 ng/kg bw per day.

6.1.2 Japan

AFB_1 dietary exposure estimates were assessed in Japan based on food consumption data from the 2005 National Health and Nutrition Survey for 2 consecutive days (17 827 individuals). Surveillance data on AFB_1 concentration levels were available from a retail food survey, with samples purchased in a random manner from local supermarkets and small retail shops in all parts of Japan from the summer of 2004 to the winter of 2006 (Sugita-Konishi et al., 2007). Foods analysed included peanut, peanut butter, chocolate, pistachio, spices, almond, job's tears tea and buckwheat. A probabilistic approach was used to simulate the dietary exposure distributions in each age group with three different scenarios of MLs of AFT in tree nuts (10, 15 and 20 µg/kg), following the same methodology as described previously for the EFSA opinion and assuming a lognormal distribution for occurrence data.

Dietary exposure estimates for AFB_1 were reported to range from 0.26 to 0.29 ng/kg bw per day at the 99.5th percentile.

6.1.3 Republic of Korea

A calculated probable daily intake of AFB_1 for people of the Republic of Korea reported by Park et al. (2004) was based on a survey for AFB_1 conducted on 88 food-grade rice samples randomly collected during July and August 2002 in Seoul and from a review of published data for food commodities from the Republic of Korea. Dietary exposure estimates for the lower- to upper-bound range were 1.19–5.79 ng/kg bw per day. It was concluded that the dietary exposure of people of the Republic of Korea to AFB_1 from rice was the major contributor to the overall dietary exposure estimate for AFB_1 in the Republic of Korea (from 75% to 93%).

6.1.4 Other countries

A review by Williams et al. (2004) described variations in mean dietary AFL intakes between countries, largely as a function of diet. Data assembled by Hall & Wild (1993) indicated that dietary exposure to AFL was 3.5–14.8 ng/kg bw per day in Kenya, 11.4–158.6 ng/kg bw per day in Swaziland, 38.6–183.7 ng/kg bw per day in Mozambique, 16.5 ng/kg bw per day in Transkei (now a region of South Africa), 4–115 ng/kg bw per day in The Gambia, 11.7–2027 ng/kg bw per day in the southern Guangxi province of China and 6.5–53 ng/kg bw per day in Thailand, whereas the exposure in the USA was lower, at 2.7 ng/kg bw per day. The exposure in Ghana, as measured from peanut consumption alone, was estimated to be 9.9–99.2 ng/kg bw per day (Awuah, 2000).

6.2 International estimates of dietary exposure from the 13 GEMS/Food Consumption Cluster Diets

International dietary exposure estimates were reported at the 1998 JECFA meeting on AFL (Annex 1, reference *132*). The evaluation reported at a later meeting in 2000 (Annex 1, reference *153*) assessed mean dietary exposure estimates for AFT for the five GEMS/Food diets from maize and groundnuts only for four ML scenarios (no ML where no samples excluded, ML of 10, 15 and 20 µg/kg for groundnuts and maize).

Dietary exposure estimates ranged from 0.56 ng/kg bw per day (in Latin America) to 11 ng/kg bw per day (in Africa) for AFT and from 0.82 ng/kg bw per day (in Europe) to 22.7 ng/kg bw per day (in Africa) for AFB_1 when no samples were excluded. When samples were excluded above 20 or 10 µg/kg, dietary exposure estimates ranged from 0.13 ng/kg bw per day (in Europe) to 1.15 ng/kg bw per day (in Africa) for AFT and from 0.3 ng/kg bw per day (in Europe) to 6.7 ng/kg bw per day (in Africa) for AFB_1.

In these estimates, AFT from groundnut consumption contributed from 0.14 to 2.7 ng/kg bw per day, and from maize consumption, from 0.35 to 8.3 ng/kg bw per day, when no samples were excluded; and AFT from groundnut consumption contributed from 0.03 to 0.18 ng/kg bw per day, and from maize consumption, from 0.17 to 1.1 ng/kg bw per day, when samples were excluded above 20 or 10 µg/kg.

Of the scenarios considered, the Committee had concluded that the greatest relative impact on estimated mean AFL levels is achieved by establishing a programme that would limit AFL contamination to less than 20 µg/kg. Depending upon assumptions made when looking at the distribution of residues, some small incremental reductions can be achieved by limiting AFL levels to no more than 15 or 10 µg/kg.

The new descriptions of the 13 GEMS/Food Consumption Cluster Diets (World Health Organization, 2006) and the actual AFL levels in foods moving in international trade considered in this assessment together provide a refinement of dietary exposure assessments for tree nuts and the relative contribution of other food sources to overall dietary exposure to AFL, which makes the diets and ML scenarios more relevant than those used for the previous evaluation by the Committee.

Tables 1 to 6 above summarize the impact of different hypothetical MLs (4, 8, 10, 15 and 20 µg/kg) for AFT on the statistical distribution of AFB_1 and AFT contents in almonds, Brazil nuts, hazelnuts, pistachios and dried figs from various export countries for 2001–2007; and the statistical distribution of AFB_1 in foods other than tree nuts contributing to the overall exposure for various countries from 2000 to 2006. These were the best available occurrence data for use in assessing international intake estimates at this meeting.

In general, the food items analysed were well characterized, and it was possible to match sources, contamination and consumption to the 13 GEMS/Food Consumption Cluster Diets (Table 8). Mean concentration data were multiplied by the total mean consumption of the corresponding food category or subcategory reported, to derive mean total dietary exposure estimates per cluster diet for AFB_1 and AFT from all food sources.

A summary of the international mean total dietary exposure estimates for AFB_1 and AFT from all tree nuts (almonds, Brazil nuts, hazelnuts and pistachios), dried figs and other contributing food sources, expressed in nanograms per kilogram body weight per day for the 13 GEMS/Food Consumption Cluster Diets, under different ML scenarios (no MLs, MLs at 4, 8, 10, 15 and 20 µg/kg) is presented in Tables 9, 10 and 11. The corresponding contributions of tree nuts, dried figs and other food sources to overall mean dietary AFT exposure (in % AFT) are also presented. In this assessment, mean lower- and upper-bound scenarios have been used in making the dietary exposure estimates employing the 13 GEMS/Food Consumption Cluster Diets.[13] The lower bound was calculated using 0 for non-detects or the LOD for trace values, whereas the upper bound was calculated using either the LOD or LOQ.

[13] Country assignments to the 13 Consumption Cluster Diets may be found at http://www.who.int/entity/foodsafety/chem/countries.pdf.

Table 8. Summary of the consumption data from 13 GEMS/Food Consumption Cluster Diets used for estimates of international dietary intake of AFB_1 and AFT

Food commodities	Consumption (g/person per day) for the 13 GEMS/Food Consumption Cluster Diets												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Tree nuts	0.0	4.7	1.5	1.0	2.7	1.1	0.0	0.2	0.0	0.0	0.1	0.3	0.7
Almonds	0.0	1.9	1.0	0.0	1.0	0.8	0.0	0.1	0.0	0.0	0.0	0.3	0.3
Brazil nuts	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
Hazelnuts	0.0	2.1	0.0	0.1	1.3	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Pistachios	0.0	0.7	0.5	0.9	0.3	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.2
Dried figs	0.0	0.6	0.4	0.0	0.2	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Dried fruits other than figs (*grapes, apricots, plums, dates and others*)	0.8	5.3	32.1	6.0	3.5	3.0	1.2	0.5	0.5	3.9	0.5	0.9	3.7
Maize	97.4	150.4	136.1	33.0	39.8	14.7	35.6	303.5	252.6	60.7	64.8	64.2	103.6
Groundnuts (*shelled and in shell*)	13.0	7.4	5.1	1.7	9.6	3.4	18.2	5.0	11.3	52.3	2.2	1.7	16.6
Other nuts (*cashews, chestnuts, walnuts, pecans*)	0.0	3.1	0.0	0.3	1.0	0.1	0.9	0.7	0.2	0.0	0.1	1.7	1.1
Oilseeds *(lin, sesame, poppy, melon, sunflower, rape*)	15.3	61.5	31.3	51.2	57.4	33.2	17.6	17.3	18.4	9.9	3.9	61.1	21.7
Cocoa products (*mass, powder, butter, other chocolate products*)	1.0	4.4	1.3	1.8	10.4	9.9	1.0	3.0	1.1	0.9	2.1	2.9	7.5
Butter of Karité nut	1.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.3	2.8	0.0	0.0	0.0
Oil of groundnut	1.7	0.8	0.5	0.1	1.4	0.4	3.0	0.3	1.5	7.9	0.3	0.0	0.4
Spices	2.7	1.1	2.4	0.9	1.8	1.1	2.3	1.9	1.4	1.3	0.4	0.6	1.7

Table 9. Mean estimates of dietary exposure to AFB_1 and AFT from all food sources for the 13 GEMS/Food Consumption Cluster Diets taking into consideration hypothetical ML scenarios for AFT (no MLs; MLs at 4, 8, 10, 15 and 20 µg/kg) in tree nuts and the contribution of tree nuts to total dietary AFT exposure

Scenario[a]		Mean dietary exposure (ng/kg bw per day) for the 13 GEMS/Food Consumption Cluster Diets												
		A	B	C	D	E	F	G	H	I	J	K	L	M
No ML	AFB_1	0.9-1.2	1.7-2.3	1.1-1.7	1.2-1.4	1.3-1.7	0.6-0.8	1.0-1.1	1.0-1.9	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.3-1.8
	AFT	1.1-1.7	2.1-3.2	1.5-2.5	1.4-1.8	1.7-2.3	0.7-1.1	1.3-1.6	1.4-2.8	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.7-2.5
	All tree nuts (% AFT)	0.0	24.6	20.0	45.0	16.8	3.7	0.0	3.3	0.0	0.0	4.3	0.8	9.3
ML 20 µg/kg	AFB_1	0.9-1.2	1.1-1.7	0.8-1.3	0.5-0.7	1.0-1.4	0.6-0.8	1.0-1.1	1.0-1.8	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.2-1.6
	AFT	1.1-1.7	1.5-2.5	1.1-2.0	0.7-1.1	1.3-2.0	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.5-2.3
	All tree nuts (% AFT)	0.0	4.7	2.6	6.3	3.2	1.6	0.0	0.3	0.0	0.0	0.6	0.3	1.1
ML 15 µg/kg	AFB_1	0.9-1.2	1.1-1.7	0.7-1.3	0.5-0.7	1.0-1.4	0.6-0.8	1.0-1.1	1.0-1.8	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.2-1.6
	AFT	1.1-1.7	1.4-2.5	1.1-2.0	0.6-1.1	1.3-2.0	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.5-2.3
	All tree nuts (% AFT)	0.0	4.1	2.2	5.0	2.8	1.5	0.0	0.3	0.0	0.0	0.5	0.3	0.9
ML 10 µg/kg	AFB_1	0.9-1.2	1.1-1.7	0.7-1.3	0.5-0.7	1.0-1.4	0.6-0.8	1.0-1.1	1.0-1.8	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.2-1.6
	AFT	1.1-1.7	1.4-2.5	1.0-2.0	0.6-1.1	1.3-2.0	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.5-2.3
	All tree nuts (% AFT)	0.0	3.3	1.7	3.6	2.3	1.4	0.0	0.2	0.0	0.0	0.4	0.2	0.7
ML 8 µg/kg	AFB_1	0.9-1.2	1.1-1.7	0.7-1.3	0.5-0.7	1.0-1.4	0.6-0.8	1.0-1.1	1.0-1.8	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.2-1.6
	AFT	1.1-1.7	1.4-2.5	1.1-2.0	0.6-1.1	1.3-2.0	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.5-2.3
	All tree nuts (% AFT)	0.0	2.9	1.5	3.0	2.1	1.2	0.0	0.2	0.0	0.0	0.4	0.2	0.7
ML 4 µg/kg	AFB_1	0.9-1.2	1.1-1.7	0.7-1.3	0.4-0.7	1.0-1.4	0.6-0.8	1.0-1.1	1.0-1.8	1.1-1.8	2.3-2.8	0.3-0.5	0.6-0.9	1.2-1.6
	AFT	1.1-1.7	1.4-2.5	1.1-2.0	0.6-1.1	1.3-2.0	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.8-1.3	1.5-2.3
	All tree nuts (% AFT)	0.0	2.3	1.1	1.9	1.7	1.1	0.0	0.1	0.0	0.0	0.4	0.2	0.5

Table 9 (contd)

[a] Lower- and upper-bound scenarios have been used in making the dietary exposure estimates for overall exposure and all tree nuts. The lower bound was calculated using 0 for non-detects or the LOD for trace values, whereas the upper bound was calculated using either the LOD or LOQ, as appropriate. “All tree nuts” includes dried figs, which contributed less than 0.3% of the dietary AFT exposure in all scenarios. % AFT is the contribution from almonds, Brazil nuts, hazelnuts, pistachios and dried figs to total dietary AFT exposure (upper-bound scenario only).

Table 10. Summary of the mean overall estimates of international dietary exposure to AFT from other contributing food sources (lower- and upper-bound scenarios) for the 13 GEMS/Food Consumption Cluster Diets and the corresponding exposure from each food commodity

		Dietary exposure to AFT (ng/kg bw per day) for 13 GEMS/Food Consumption Cluster Diets												
		A	B	C	D	E	F	G	H	I	J	K	L	M
Overall exposure from other sources		1.1-1.7	1.3-2.4	1.0-2.0	0.6-1.0	1.3-1.9	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.7-1.3	1.5-2.2
Mean dietary exposure to AFT from individual food sources[a]	Maize	0.2-0.7	0.4-1.0	0.3-0.9	0.1-0.2	0.1-0.3	0.04-0.10	0.1-0.2	0.8-2.1	0.6-1.7	0.2-0.4	0.2-0.5	0.2-0.4	0.3-0.7
	Groundnuts	0.7-0.7	0.4-0.4	0.3-0.3	0.1-0.1	0.5-0.5	0.2-0.2	0.9-1.0	0.3-0.3	0.6-0.6	2.6-2.9	0.1-0.1	0.1-0.1	0.8-0.9
	Oilseeds	0.1-0.2	0.4-0.6	0.2-0.3	0.3-0.5	0.4-0.6	0.2-0.3	0.1-0.2	0.1-0.2	0.1-0.2	0.1-0.1	0.02-0.04	0.4-0.6	0.1-0.2
	Cocoa products	0.02-0.04	0.1-0.2	0.03-0.1	0.04-0.1	0.2-0.4	0.2-0.4	0.02-0.04	0.1-0.1	0.03-0.04	0.02-0.03	0.1-0.1	0.1-0.1	0.2-0.3
	Other nuts	0.0-0.0	0.04-0.1	0.0-0.0	0.0-0.01	0.01-0.02	0.0-0.0	0.01-0.02	0.01-0.01	0.0-0.0	0.0-0.0	0.0-0.0	0.02-0.03	0.01-0.02
	Dried fruits other than figs	0.0-0.01	0.02-0.1	0.1-0.3	0.02-0.1	0.01-0.03	0.01-0.03	0.0-0.01	0.0-0.0	0.0-0.0	0.01-0.04	0.0-0.0	0.0-0.01	0.01-0.04
	Butter of Karité nut	0.01-0.02	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.02	0.0-0.0	0.0-0.0	0.0-0.0
	Peanut oil	0.02-0.03	0.01-0.01	0.01-0.01	0.0-0.0	0.02-0.02	0.01-0.01	0.04-0.05	0.0-0.0	0.02-0.02	0.1-0.1	0.0-0.0	0.0-0.0	0.01-0.01
	Spices	0.07-0.08	0.03-0.03	0.07-0.07	0.02-0.03	0.1-0.1	0.03-0.03	0.1-0.1	0.1-0.1	0.04-0.04	0.04-0.04	0.01-0.01	0.02-0.02	0.1-0.1

[a] Lower- and upper-bound scenarios have been used in making the dietary exposure estimates for overall exposure and individual food sources. The lower bound was calculated using 0 for non-detects or the LOD for trace values, whereas the upper bound was calculated using either the LOD or LOQ, as appropriate.

Table 11. Mean estimates of dietary exposure to AFT from almonds, Brazil nuts, hazelnuts and pistachios and for the 13 GEMS/Food Consumption Cluster Diets, taking into consideration the impact of different hypothetical ML scenarios for AFT (no MLs; MLs of 4 and 20 µg/kg) in tree nuts

		Dietary exposure to AFT (ng/kg bw per day) for 13 GEMS/Food Consumption Cluster Diets												
		A	B	C	D	E	F	G	H	I	J	K	L	M
Overall exposure from other sources[a]		1.1-1.7	1.3-2.4	1.0-2.0	0.6-1.0	1.3-1.9	0.7-1.1	1.3-1.6	1.3-2.7	1.4-2.7	3.0-3.7	0.4-0.7	0.7-1.3	1.5-2.2
Scenario[b]														
No ML	All tree nuts	0.0-0.0	0.8-0.8	0.5-0.5	0.8-0.8	0.4-0.4	0.04-0.04	0.0-0.0	0.1-0.1	0.0-0.0	0.0-0.0	0.03-0.03	0.01-0.01	0.2-0.2
	Pistachios (% AFT)	0.0	20.1	18.4	44.8	12.0	0.0	0.0	3.2	0.0	0.0	0.0	0.0	7.4
	Other tree nuts (% AFT)	0.0	4.4	1.7	0.2	4.9	3.7	0.0	0.1	0.0	0.0	4.3	0.8	1.9
ML 20 µg/kg	All tree nuts	0.0-0.0	0.1-0.1	0.1-0.1	0.1-0.1	0.1-0.1	0.02-0.02	0.0-0.0	0.01-0.01	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.03-0.03
	Pistachios (% AFT)	0.0	2.0	1.8	6.1	1.1	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.6
	Other tree nuts (% AFT)	0.0	2.7	0.8	0.2	2.1	1.6	0.0	0.0	0.0	0.0	0.6	0.3	0.5
ML 4 µg/kg	All tree nuts	0.0-0.0	0.1-0.1	0.02-0.02	0.02-0.02	0.03-0.03	0.01-0.01	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.0-0.0	0.01-0.01
	Pistachios (% AFT)	0.0	0.6	0.5	1.8	0.3	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.2

Table 11 (contd)

	Dietary exposure to AFT (ng/kg bw per day) for 13 GEMS/Food Consumption Cluster Diets												
	A	B	C	D	E	F	G	H	I	J	K	L	M
Other tree nuts (% AFT)	0.0	1.7	0.6	0.1	1.4	1.1	0.0	0.0	0.0	0.0	0.4	0.2	0.3

[a] Mean concentration as reported for other contributing food sources to the overall dietary exposure to AFT and the corresponding food consumption from the 13 GEMS/Food Consumption Cluster Diets.

[b] Lower- and upper-bound scenarios have been used in making the dietary exposure estimates for overall exposure and all tree nuts. The lower bound was calculated using 0 for non-detects or the LOD for trace values, whereas the upper bound was calculated using either the LOD or LOQ, as appropriate. "All tree nuts" includes dried figs, which contributed less than 0.3% of the dietary AFT exposure in all scenarios. % AFT is the contribution from almonds, Brazil nuts, hazelnuts, pistachios and dried figs to the total dietary AFT exposure (upper-bound scenario only).

6.2.1 *Estimates of overall dietary exposure to aflatoxin—scenario with no MLs*

International mean dietary exposure estimates for AFT from all sources were estimated to range from 0.4–0.7 ng/kg bw per day (cluster K) to 3.0–3.7 ng/kg bw per day (cluster J) for the 13 GEMS/Food Consumption Cluster Diets, by assuming a body weight of 60 kg and using the lower-bound/upper-bound approach. In these estimates, mean dietary AFB_1 exposures ranged from 0.3–0.5 ng/kg bw per day to 2.3–2.8 ng/kg bw per day for the same clusters (Table 9).

In these estimates, dietary exposures to AFT from maize, groundnuts, oilseeds and cocoa products made the greatest contribution to total exposure in all cluster diets (Table 10):

- Maize ranged from 0.04–0.10 ng/kg bw per day (cluster F) to 0.8–2.1 ng/kg bw per day (cluster H).
- Groundnuts ranged from 0.1 ng/kg bw per day (clusters D and L) to 2.6–2.9 ng/kg bw per day (cluster J).
- Oilseeds ranged from 0.02–0.04 ng/kg bw per day (cluster K) to 0.4–0.6 ng/kg bw per day (cluster B).
- Cocoa products ranged from 0.02–0.03 ng/kg bw per day (cluster J) to 0.2–0.4 ng/kg bw per day (clusters E and F).

6.2.2 *Dietary exposure estimates for tree nuts and dried figs*

The mean contribution to dietary AFT exposure from consumption of almonds, Brazil nuts, hazelnuts, pistachios and dried figs ranged from 0 ng/kg bw per day (clusters A, G, I and J; nut consumption reported as zero for these clusters) up to 0.8 ng/kg bw per day (clusters B and D). In five cluster diets (B, C, D, E and M), the contribution from almonds, Brazil nuts, hazelnuts, pistachios and dried figs was higher than 5% of the overall dietary exposure to AFT (Table 9). Mean dietary exposures for all other cluster diets from tree nuts (including dried figs) were below 0.1 ng/kg bw per day.

Pistachios were the main contributor to dietary AFT exposure from tree nuts in these five cluster diets with higher than 5% contribution to overall dietary AFT exposure, ranging from 0.2 to 0.8 ng/kg bw per day, equivalent to 7–45% of the total AFT from all sources (Table 11). Almonds, Brazil nuts and hazelnuts contributed up to 0.1 ng/kg bw per day, and dried figs less than 0.01 ng/kg bw per day, in all Cluster Consumption Diets.

6.2.3 *Effect of hypothetical MLs in almonds, Brazil nuts, hazelnuts, pistachios and dried figs on dietary exposure*

The Committee evaluated the impact on dietary exposure to AFT of setting hypothetical MLs of 4, 8, 10, 15 or 20 µg/kg for AFT in almonds, Brazil nuts, hazelnuts, pistachios and dried figs. For dried figs and tree nuts other than pistachios, the contribution to total dietary AFT exposure is less than 5%, regardless of whether an ML is in place or not. This is explained by the fact that the main part of the dietary exposure to AFT comes from other food sources (Tables 2, 3, 4 and 5).

Using the five cluster diets where almonds, Brazil nuts, hazelnuts, pistachios and dried figs contribute more than 5% to dietary AFT exposure (clusters B, C, D, E and M), and assuming a body weight of 60 kg, the Committee estimated that an enforced ML of 20, 15, 10, 8 or 4 µg/kg results in dietary exposures to AFT ranging from 0.12, 0.10, 0.08, 0.07 and 0.06 ng/kg bw per day in the cluster with the highest exposure (B) to 0.03, 0.02, 0.02, 0.02 and 0.01 ng/kg bw per day in the cluster with the lowest exposure (M).

United Kingdom food consumption data for vegetarians and vegans showed that for high-level consumers of almonds, Brazil nuts, hazelnuts and pistachios, enforcing an ML of 20 µg/kg reduces total dietary AFT exposure when compared with no ML. Setting a lower ML would have little impact compared with the ML of 20 µg/kg. The dietary exposure from tree nuts assuming no ML was estimated to be 5.8 ng/kg bw per day. The estimate with an ML of 20 µg/kg would be 0.5 ng/kg bw per day and with an ML of 4 µg/kg would be 0.2 ng/kg bw per day.

In these analyses, the contribution from tree nuts to the total dietary AFT exposures in all five cluster diets, whatever the ML scenario (4, 8, 10, 15 or 20 µg/kg), will remain below a dietary exposure of 0.1 ng/kg bw per day compared with less than 0.8 ng/kg bw per day for the scenario with no MLs. The highest decrease in AFT exposure results from the contribution from pistachios to total dietary AFT exposure when setting an ML at 20 µg/kg in comparison with no MLs.

The Committee also noted that in all these different ML scenarios, dried figs were included (dietary exposures not shown in tables). However, the contribution of dried figs (less than 0.01 ng/kg bw per day) to total dietary AFT exposure estimates in all Consumption Cluster Diets, whatever the ML scenario, would be less than 0.3% of the overall dietary AFT exposure.

The Committee noted the previous assessments of exposure to AFT made by JECFA in 1998 (Annex 1, reference *132*) and EFSA in 2007 (European Food Safety Authority, 2007). The estimates made at this meeting for EU dietary exposures—0.7–2.5 ng/kg bw per day for clusters B, E and F (with ML scenario from 4 to 20 µg/kg for tree nuts)—were in the range of those reported in the EFSA opinion: 1.0–2.5 ng/kg bw per day (with ML scenario from 4 to 10 µg/kg for tree nuts, and including high-level consumers of these nuts). These estimates can be compared with the JECFA estimate of 0.8 ng/kg bw per day (with ML scenario from 10 to 20 µg/kg in groundnuts) (Annex 1, reference *132*). In these estimates, groundnuts and maize were the main contributors to AFT exposure, ranging from 0.2 to 1.4 ng/kg bw per day at the current meeting, compared with 1.1–2.0 ng/kg bw per day in the JECFA assessment (Annex 1, reference *132*) and 0.03–1.0 ng/kg bw per day in the EFSA opinion (European Food Safety Authority, 2007).

7. PREVENTION AND CONTROL OF AFLATOXIN PRODUCTION

A prevention programme to reduce and control AFL contamination should be established, considering various steps from cultivation through harvesting, post-harvesting, processing, storage, transportation and marketing (Campbell et al., 2003, 2005; Kabak et al., 2006).

7.1 Aflatoxin-producing fungi

AFL are found in different tree nuts as a result of fungal contamination both pre- and post-harvest, with the rate and degree of contamination dependent on tree or shrub species, geographical location, meteorological conditions, and different harvest, drying, processing and storage conditions, among others (Pitt, 2006).

AFL production has been reported in a large number of fungi, but *Aspergillus flavus* and *A. parasiticus* were the only species reliably reported to accumulate AFL. *Aspergillus flavus* is ubiquitous, favouring the aerial parts of plants (leaves, flowers), and produces B AFL. *Aspergillus parasiticus* produces both B and G AFL, is more adapted to a soil environment and has more limited distribution. Later, *A. nomius*, *A. toxicarius*, *A. pseudotamarii*, *A. flavus* var. *columnaris*, *A. flavus* var. *parvisclerotigenus*, *A. zhaoqingensis* and *A. bombycis* from section *Flavi* were also reported as AFL producers (Klich et al., 2000; Ito et al., 2001; Peterson et al., 2001; Frisvad et al., 2006; Pitt, 2006). Other species in *Aspergillus* and one of its teleomorphs, *Emericella*, but also species in *Monocillium*, *Chaetomium*, *Bipolaris* and *Humicola*, are able to produce sterigmatocystin, an AFL precursor (Frisvad & Samson, 2004; Frisvad et al., 2004, 2005). These species were screened for AFL, but AFL were not found in these other genera. However, AFL were discovered in the section *Ochraceorosei* in *Aspergillus* (*A. ochraceoroseus* and *A. rambellii*) and in three species of *Emericella*: *E. astellata*, *E. venezuelensis* and *E. olivicola* (Klich et al., 2003; Cary et al., 2005). The latter five species accumulate both AFB_1 and sterigmatocystin, in contrast to species in section *Flavi*, which accumulate AFL only and are particularly efficient producers of 3-*O*-methylsterigmatocystin. None of the latter five species, with the possible exception of *E. olivicola*, seems to be of significance for food safety. This leaves members of *Aspergillus* section *Flavi* as the important AFL producers in foods and foodstuffs. In some cases, the nomenclature was updated, and the following species are considered to be important AFL producers in special situations: *A. parvisclerotigenus*, *A. nomius*, *A. toxicarius*, *A. pseudotamarii* and *A. bombycis* (Cotty & Cardwell, 1999; Freire et al., 2000; Ehrlich et al., 2007). Several papers on the molecular biology of AFL producers indicate that more species exist in the section *Flavi* (Färber et al., 1997; Pitt & Samson, 2000; Cary & Ehrlich, 2006).

The occurrence of these fungal species varies by food commodity and geographically (Abdel-Hafez & Saber, 1993; Doster & Michailides, 1994, 1995; Doster et al., 1996; Freire et al., 2000; Moretti et al., 2000; Hua & McAlpin, 2001; Bayman et al., 2002; Simekş et al., 2002; Logrieco et al., 2003; Iamanaka et al., 2005, 2007; Gürses, 2006; Codex Committee on Contaminants in Food, 2007a). Brazil nuts and dried figs seemed to have a different AFL profile from the rest of the analysed food products. The calculated relationships from the submitted data seem to indicate that there are atypical *Aspergillus* isolates on dried figs, as reported by Steiner et al. (1988).

7.2 Pre-harvest control

The main ways to reduce AFL contamination are to control the presence of insects such as the orangeworm, *Amyelois transitella*, in almonds and in pistachio

nuts and other pests in the orchard, minimize early split nut formation and avoid late harvesting (Schatzki & Ong, 2000, 2001; Doster et al., 2001b; Michailides, 2005). Research in the use of sex pheromones for insect control to replace pesticides is increasing in response to food safety concerns (Campbell et al., 2005). Additional non-pesticidal approaches include augmenting the constitutive natural products, as, for example, in the case of almonds, which deter insect feeding (Dicenta et al., 2002), or the use of natural antifungal products (Campbell et al., 2005; Kabak et al., 2006). Pistachio shell splitting is sensitive to irrigation deficits; therefore, it should be carefully monitored (Ferguson et al., 2005). Delaying harvest allows more time for AFL-producing fungi, and it can also increase insect attack (Campbell et al., 2003; Bentley et al., 2005; Michailides, 2005). In Iran, the most effective ways to reduce the AFL content of pistachio nuts were the introduction of early harvest and keeping the harvesting period and drying time as short as possible.

A possible preventive treatment is the application of microorganisms (Doster et al., 2001a; Hua, 2002, 2004; Palumbo et al., 2006). In the last few years, experiments have been performed using atoxigenic strains of *A. flavus* to control AFL contamination in pistachios (Michailides, 2005) and in figs (Doster et al., 2001a). The potential of saprophytic yeast as a biocontrol agent has been investigated by Hua (2002, 2004). It is evident, though, that further research is needed to determine if these suggested practices are able to reduce mycotoxin contamination.

A major reservoir of *Aspergillus* spores in tree nuts can occur in the leaf, hull and unharvested litter surrounding the trees. This type of litter presents a special problem to tree nuts in general, but especially to Brazil nuts when they are harvested after they have fallen to the jungle floor. In many cases, they are in direct contact with the soil for several days prior to collection (Doster & Michailides, 1984; Arrus et al., 2005). It would be interesting to try to develop an improved way of collection aimed at reducing the inoculum sources and AFL contamination as much as possible and minimizing the risk to collectors when remaining pods fall down after the crop season.

7.3 Genetic improvement

Some procedures used to reduce and prevent AFL production include a selection of resistant varieties. Progress has been made in all crops, and genetic improvement offers considerable potential. Current status and prospects for the future are discussed by Mehlenbacher (2003) for each tree nut crop, including efforts at mapping, marker-assisted selection and transformation (Gradziel & Dandekar, 2001), and information on fig selection by Doster et al. (2001a). The small number and size of breeding programmes are major limitations to genetic improvement of tree nuts.

7.4 Post-harvest control

Different oil contents of tree nuts emphasize the necessity of using water activity as a conservation parameter instead of moisture content (Bianco et al., 2001) and should be carefully controlled during storage. Collection of useful data

for future modelling that integrates technological and practical achievements requires knowledge of not only fungal distribution in each product and in the different steps of the food-chain, but also the fungal growth and AFL production kinetics during the storage period in relation to weather conditions or the storage parameters.

Some results showed that pistachio AFL contamination in storage can be controlled by oxygen exclusion (Iqbal et al., 2006).

7.5 Physical decontamination

Removal of AFL-contaminated nuts or figs by means of physical segregation is the most effective control measure for reducing levels of AFL in a lot to an acceptable level.

Some adsorbents can bind AFL and thus remove them from aqueous solutions. Natrolite ($Na_{16}[(AlO_2)_{16}(SiO_2)_{24}]\cdot 16H_2O$) was recently shown to decontaminate pistachio nuts, reducing AFB_1 with a slurry of 5% concentration, but its efficacy against AFB_2 is proving to be limited (Fooladi & Farahnaky, 2003).

The complete elimination of AFL contamination in the evaluated products is currently not realistically achievable, and research should be improved to develop further detoxification strategies.

8. COMMENTS

8.1 Analytical methods

In the studies evaluated by the Committee at its present meeting, it was usually clear which AFT analytical method had been used. However, in the submitted data, detection and quantification limits for AFT were calculated in different ways. One method defined the LOD of AFT as twice the value of the LOD of AFB_1, whereas the second used the sum of the LODs of AFB_1, AFB_2, AFG_1 and AFG_2. The Committee concluded that both definitions overestimate the LOD of AFT, resulting in conservative estimates of the exposure to AFT for the upper-bound estimate. The Committee also concluded that it was better to restrict data used in the dietary exposure assessment to those with validated recoveries greater than 70% than to correct for lower recoveries. The Committee also noted that surveillance data should be accompanied by a clear description of the analytical method used; recoveries of the analytical methodology chosen should be specific to the food matrix tested; and LODs and LOQs should be provided with the definitions used to derive them. Efforts should be made to harmonize the nomenclature and the methodologies by which the LOD and LOQ were calculated.

The combination of liquid chromatography with mass spectrometry is one useful technique for the confirmation of the presence of AFL in foodstuffs. Although some methods are already implemented in routine analysis, the limited number of reference materials, high investment costs and lack of the required sensitivity could be a barrier to use for AFL surveillance, because it was noted that for accurate

dietary exposure assessments, the LOD/LOQ should be as low as technically possible. This is due to the fact that many foods that might be expected to contain AFL do not contain *detectable* AFL contamination, and the default value assigned to those censored samples will affect the estimated dietary exposures (upper-bound estimates only).

8.2 Sampling protocols

Almost all of the submitted data on AFT were derived using sampling plans designed for regulatory purposes. The producing countries that submitted data to the Committee presented sampling plans similar to or the same as those following EC No. 401/2006 for the determination of AFL, which includes edible nuts and dried figs. It was observed that in some producing countries, there are two sampling plans: one for commodity to be exported to the EU, and the second for commodity to be exported to other countries with less strict regulations. There remains a need for harmonized sampling plans, both between different countries and within the same country. The Committee noted that AFL sampling plans should be determined by data relating to contamination distributions and uncertainties within the particular foodstuff. The resulting knowledge of the uncertainty among sample test results should allow each country to refine its sampling plans using, for example, larger sample sizes and/or fewer analytical repetitions in order to meet harmonized criteria. The Committee noted that the data received for this analysis were robust.

8.3 Effects of processing

Although AFL are highly stable, studies have indicated that they are degraded in contaminated food by heat treatment. For example, the roasting of pistachio nuts at 150 ºC for 30 min reduced AFT levels by 63% when the initial level was 44 µg AFB_1/kg, 24% when the initial level was 213 µg AFB_1/kg, 17% when the initial level was 21.9 µg AFB_1/kg and 47% when the initial level was 18.5 µg AFB_2/kg.

8.4 Aflatoxin occurrence and levels in food commodities and the potential effect of MLs in almonds, Brazil nuts, hazelnuts, pistachios and dried figs

AFL occurrence data on almonds, Brazil nuts, hazelnuts, pistachios and dried figs were obtained from both producing and importing countries. The Committee decided to base the assessment of the impact of different MLs for AFT for almonds, Brazil nuts, hazelnuts, pistachios and dried figs (4, 8, 10, 15 and 20 µg/kg) on data provided by producing countries, as these are more likely to represent the actual occurrence of AFL in the commodities. The primary producing countries (FAOSTAT, 2007) were, for almonds, the USA (42% of the world market); for Brazil nuts, Latin America (100%); for hazelnuts, Turkey (70%); for pistachios, the Islamic Republic of Iran (65%); and for dried figs, Turkey (63% for dried fruits). Turkey is the primary producing country for hazelnuts, but the Committee received no data on AFT levels in hazelnuts from Turkey; therefore, the Committee chose to use all of the submitted data supplied by the EU, the USA and Japan for its analyses.

The mean concentrations of AFT in nuts and dried figs in the main producing countries were, for almonds, 2 µg/kg; for Brazil nuts, 20 µg/kg; for hazelnuts, 2 µg/kg; for pistachios, 54 µg/kg; and for dried figs, 1 µg/kg. The effects of the theoretical full enforcement of MLs (all samples above the ML would be excluded from the distribution) at 20, 15, 10, 8 and 4 µg/kg are shown in Table 6. The reductions in mean AFT concentrations would be approximately 2- to 3-fold for almonds, 10-fold for Brazil nuts, 2- to 4-fold for hazelnuts, 10- to 50-fold for pistachios and 2-fold for dried figs. The corresponding proportion of rejected samples would be 1–3% for almonds, 11–17% for Brazil nuts, 1–7% for hazelnuts, 40–60% for pistachios and 1–3% for dried figs.

8.5 Assessment of dietary exposure

At the regional level, published studies reported that estimated mean dietary exposures to AFT for the general population from all food sources were 0.93–2.4 ng/kg bw per day in Europe, 3.5–180 ng/kg bw per day in Africa, 0.3–53 ng/kg bw per day in Asia and 2.7 ng/kg bw per day in the USA.

In this assessment, mean lower- and upper-bound scenarios have been used in making the dietary exposure estimates employing the 13 GEMS/Food Consumption Cluster Diets (Tables 8, 9, 10 and 11). The lower bound was calculated using 0 for non-detects or the LOD for trace values, whereas the upper bound was calculated using either the LOD or LOQ, as appropriate.

The Committee employed the 13 GEMS/Food Consumption Cluster Diets to make international estimates of dietary AFT exposure from all sources. These were estimated to range from 0.4–0.7 ng/kg bw per day (cluster K) to 3.0–3.7 ng/kg bw per day (cluster J), by assuming a body weight of 60 kg and using the lower-bound/upper-bound approach. The mean total dietary exposure to AFT from maize, groundnuts, oilseeds and cocoa products made the greatest contribution to total exposure in all cluster diets (Table 10). Dietary AFB_1 exposure ranged from 0.3–0.5 ng/kg bw per day to 2.3–2.8 ng/kg bw per day for the same clusters (Table 9).

8.5.1 Almonds, Brazil nuts, hazelnuts, pistachios and dried figs

The mean contribution to dietary AFT exposure from consumption of almonds, Brazil nuts, hazelnuts, pistachios and dried figs ranged from 0 ng/kg bw per day (clusters A, G, I and J; nut consumption reported as zero for these clusters) up to 0.8 ng/kg bw per day (clusters B and D). In five cluster diets (B, C, D, E and M), the contribution from almonds, Brazil nuts, hazelnuts and pistachios was higher than 5% of the overall dietary exposure to AFT (Table 9).

Pistachios were the main contributor to dietary AFT exposure from tree nuts in all five cluster diets, ranging from 0.2 to 0.8 ng/kg bw per day, equivalent to 7–45% of the total AFT from all sources (Table 11). Almonds, Brazil nuts and hazelnuts contributed up to 0.1 ng/kg bw per day, and dried figs less than 0.01 ng/kg bw per day, in all Consumption Cluster Diets.

8.5.2 Foods other than tree nuts and dried figs

In order to evaluate the relative contribution of tree nuts and dried figs to the overall AFT exposure, the Committee considered other foods known to contribute to the overall exposure to AFT in humans. Occurrence data and dietary exposures to AFT from these other foods were described. Food commodities included in the mean overall exposure were maize, groundnuts (i.e. peanuts) and other nuts (i.e. walnuts, cashews, chestnuts, macadamia nuts, pecans), dried fruits other than figs (apricots, plums, grapes, dates and others), spices, cocoa and cocoa products (cocoa mass, cocoa butter, cocoa powder), peanut butter, peanut cream, oilseeds and butter of Karité nut.

The majority of the data included in the estimation of dietary AFT exposure from other food sources came from the EU. The Committee noted that the European data do not reflect the actual mean values in other world regions for some foods considered here, as the mean concentration of AFT in the EU takes into account fewer highly contaminated samples due to existing EU MLs compared with regions with higher MLs or lack of enforcement.

The mean concentrations of AFB_1 and AFT were less than 1 µg/kg for most foods, except spices, cocoa products, groundnuts and butter of Karité nut, where mean levels ranged between 2 and 4 µg/kg.

The Committee noted that different concentrations in rice were reported in different regions (producing and non-producing countries), with mean AFT levels around 0.6–1.0 µg/kg in the EU, 0.2–1.2 µg/kg in the Republic of Korea and 0.1–0.2 µg/kg in Qatar, with no reports of detected levels in other regions, including Japan and Argentina. High AFT levels, such as those for peanuts or maize, have never been reported in rice; the highest reliably reported levels are less than 10 µg/kg. Because of uncertainties in the data, rice was not included in estimating overall dietary exposures to AFT for comparison with the contribution from almonds, Brazil nuts, hazelnuts, pistachios and dried figs. In regions where rice is a major component of the diet, any low levels of AFT in rice may lead to its being a major contributor to total dietary exposure to AFT, even though that exposure may be low when compared with that in other regions.

8.6 Effect of hypothetical MLs in almonds, Brazil nuts, hazelnuts, pistachios and dried figs on dietary exposure

The Committee evaluated the impact on dietary exposure to AFT of setting hypothetical MLs of 4, 8, 10, 15 or 20 µg/kg for AFT in almonds, Brazil nuts, hazelnuts, pistachios and dried figs. For dried figs and tree nuts other than pistachios, the contribution to total dietary AFT exposure is less than 5%, regardless of whether an ML is in place or not. This is explained by the fact that the main part of the dietary exposure to AFT comes from other food sources (Tables 2–6).

Using the five cluster diets where almonds, Brazil nuts, hazelnuts, pistachios and dried figs contribute more than 5% to dietary AFT exposure (clusters B, C, D, E and M), and assuming a body weight of 60 kg, the Committee estimated that an enforced ML of 20, 15, 10, 8 or 4 µg/kg results in dietary exposures to AFT ranging

from 0.12, 0.10, 0.08, 0.07 and 0.06 ng/kg bw per day in the cluster with the highest exposure (D) to 0.03, 0.02, 0.02, 0.02 and 0.01 ng/kg bw per day in the cluster with the lowest exposure (M).

United Kingdom food consumption data for vegetarians and vegans showed that for high-level consumers of almonds, Brazil nuts, hazelnuts and pistachios, enforcing an ML of 20 µg/kg reduces total dietary AFT exposure when compared with no ML. Setting a lower ML would have little impact compared with the ML of 20 µg/kg. The dietary exposure from tree nuts assuming no ML was estimated to be 5.8 ng/kg bw per day. The estimate with an ML of 20 µg/kg would be 0.5 ng/kg bw per day, and with an ML of 4 µg/kg would be 0.2 ng/kg bw per day.

In these analyses, the contribution from tree nuts to the total dietary AFT exposures in all five cluster diets, whatever the ML scenario (4, 8, 10, 15 or 20 µg/kg), will remain below 0.1 ng/kg bw per day, compared with <0.8 ng/kg bw per day for the scenario with no MLs. The highest decrease in AFT exposure results from the contribution from pistachios to total dietary AFT exposure when setting an ML at 20 µg/kg in comparison with no ML.

The Committee also noted that in all these different ML scenarios, dried figs were included (dietary exposures not shown in tables). However, the contribution of dried figs (<0.01 ng/kg bw per day) to total dietary AFT exposure estimates in all Consumption Cluster Diets, whatever the ML scenario, would be less than 0.3% of the overall dietary AFT exposure.

The Committee noted the previous assessments of exposure to AFT made by JECFA in 1998 (Annex 1, reference *132*) and EFSA in 2007 (European Food Safety Authority, 2007). The estimates made at the present meeting for EU dietary exposures (0.7–2.5 ng/kg bw per day for European clusters B, E and F, with MLs from 4 to 20 µg/kg for tree nuts) were in the range of those reported in the EFSA opinion, where AFT exposures ranged from 1.0 to 2.5 ng/kg bw per day (with MLs from 4 to 10 µg/kg for tree nuts, and including high-level consumers of these nuts), compared with 0.8 ng/kg bw per day reported by JECFA in 1998 (with MLs from 10 to 20 µg/kg in groundnuts). In these estimates, groundnuts and maize were the main contributors to AFT exposure, ranging from 0.2 to 1.4 ng/kg bw per day at the current meeting, compared with 1.1–2.0 ng/kg bw per day in the 1998 JECFA evaluation and 0.03–1.0 ng/kg bw per day in the EFSA opinion.

9. EVALUATION

The Committee noted that the majority of data included in the estimation of dietary AFT exposure from foods other than almonds, Brazil nuts, hazelnuts, pistachios and dried figs came from the EU and that these data do not reflect the actual mean values in other world regions. This probably results in an underestimate of dietary AFT exposure and overstates the relative contribution of dietary AFT exposure from tree nuts. The Committee decided to base the assessment of the impact of different MLs for AFT on data provided by producing countries, noting that these better represent the materials in commerce and result in a robust estimate of dietary AFT exposure from tree nuts.

The Committee calculated that the consumption of almonds, Brazil nuts, hazelnuts, pistachios and dried figs contributes greater than 5% of the dietary AFT exposure in only five cluster diets (clusters B, C, D, E and M). If fully enforced, an ML at 20 µg/kg in almonds, Brazil nuts, hazelnuts, pistachios and dried figs would have an impact on the relative contribution to dietary AFT exposure only in these clusters, including high-level consumers of the tree nuts. This is due solely to the elevated AFT level in pistachios. For the tree nuts other than pistachios, the presence of an ML has no effect on dietary AFT exposure.

Moreover, the Committee concluded that enforcing an ML of 15, 10, 8 or 4 µg/kg would have little further impact on the overall dietary exposure to AFT in all five of the highest exposed population groups, compared with setting an ML of 20 µg/kg. The proportion of rejected samples from the world market would be between 1% (ML 20 µg/kg) and 3% (ML 4 µg/kg) for almonds, 11% and 17% for Brazil nuts, 1% and 7% for hazelnuts and 40% and 60% for pistachios, respectively.

Based on the large data sets on AFT concentrations in dried figs submitted at this meeting by Turkey, the most important producing country for dried figs (>40 000 data points), the Committee concluded that whatever the hypothetical ML scenario applied (no ML, 4, 8, 10, 15 or 20 µg/kg) to dried figs, there would be no impact on the overall dietary exposure to AFT (below 0.03%, equivalent to a dietary exposure of <0.01 ng/kg bw per day), and that the proportion of rejected samples from the world market could range between 1% and 3% for MLs at 20 µg/kg and 4 µg/kg, respectively.

The Committee noted that the reduction of dietary AFT exposure is an important public health goal, particularly in populations that consume high levels of any potentially AFT-contaminated food.

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OCHRATOXIN A (addendum)

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1. EXPLANATION

Ochratoxin A was first evaluated by the Committee at its thirty-seventh meeting (Annex 1, reference *94*). The key adverse effects noted involved toxicity to the kidney. The Committee established a provisional tolerable weekly intake (PTWI) of 112 ng/kg body weight (bw), on the basis of deterioration of renal function in pigs, for which the lowest-observed-effect level (LOEL) was 8 µg/kg bw per day, and application of a safety factor of 500. At that time, the Committee recommended that further studies be conducted to elucidate the role of ochratoxin A in causing nephropathy in pigs, the mode of action of ochratoxin A as a kidney carcinogen in rodents and the possible role of ochratoxin A in human disease.

Ochratoxin A was re-evaluated by the Committee at its forty-fourth meeting (Annex 1, reference *116*), when it considered toxicological data that had become available since the previous evaluation, including epidemiological studies of nephropathy, genotoxicity studies and studies on experimental nephrotoxicity. At that meeting, the Committee reconfirmed the PTWI, rounding it to 100 ng/kg bw, and reiterated its request for further studies on ochratoxin A.

Ochratoxin A was again evaluated by the Committee at its fifty-sixth meeting (Annex 1, reference *152*), when it was noted that the adverse effect at the lowest effective dose in several mammalian species was nephrotoxicity and that this was likely to be true in humans as well. Although an association between the intake of ochratoxin A and nephropathy in humans had been postulated, causality had not been established. Concerning carcinogenicity, the Committee concluded that the new toxicological data available since the previous evaluation raised further questions about the mode of action of ochratoxin A. Both genotoxic and non-genotoxic modes of action had been proposed. The Committee noted that further studies to address these issues were in progress and retained the previously established PTWI of 100 ng/kg bw, pending the results of these studies.

New data from surveys of food commodities for contamination with ochratoxin A were also considered, and intakes were estimated for various countries and regions of the world. From estimates based mainly on European data, the Committee noted that the intake of ochratoxin A by 95th percentile consumers of cereals may approach the PTWI from this source alone. Given the distribution of contamination of cereals with ochratoxin A, the Committee concluded that application of a maximum limit (ML) of 5 or 20 µg/kg would make no significant difference to the average intake. Efforts were needed to ensure that intakes of ochratoxin A did not exceed the PTWI, and the Committee considered that this could

best be achieved by lowering overall contamination by appropriate agricultural, storage and processing practices.

At the thirty-eighth session of the Codex Committee on Food Additives and Contaminants (CCFAC) (Codex Alimentarius Commission, 2006), the Committee was again asked to re-evaluate ochratoxin A, considering all the data available on toxicology and exposure assessment, particularly data from developing countries, including the impact of different MLs for cereals (5 or 20 µg/kg) and effects of processing on residual levels in foods.

For this evaluation, the Committee considered new toxicological studies that had become available since the last evaluation; these included further studies on developmental toxicity, neurotoxicity, immunotoxicity, nephrotoxicity and genotoxicity and studies on the mode of action of ochratoxin A in the kidney. The Committee also considered the opinion on ochratoxin A in human food published by the European Food Safety Authority (EFSA) in 2006 (European Food Safety Authority, 2006). New data on analytical methods, sampling protocols and the effects of processing were also considered, together with methods of prevention and control and levels and patterns of food contamination. A new dietary exposure assessment was conducted, and the impact of different MLs for cereals was considered.

2. BIOLOGICAL DATA

In this addendum, relevant findings for risk assessment published since the Committee's last evaluation (Annex 1, reference *153*) are described. In addition, for aspects critical to the current re-evaluation (in particular, the mode of action of ochratoxin A as a kidney carcinogen), some of the data evaluated previously by this Committee are briefly summarized in order to set the context for the more recent information.

2.1 Biochemical aspects

2.1.1 Absorption, distribution, metabolism and excretion

The absorption, distribution, metabolism and excretion of ochratoxin A have been summarized previously by the Committee as follows (Annex 1, reference *153*). Ochratoxin A is efficiently absorbed from the gastrointestinal tract, mainly in the small intestine. Information from a number of species shows that it is distributed via the blood, mainly to the kidneys, with lower concentrations being found in liver, muscle and fat. Transfer to milk has been demonstrated in rats, rabbits and humans, but little is transferred to the milk of ruminants, owing to hydrolysis of ochratoxin A into phenylalanine and ochratoxin *alpha* by the rumenal microflora. The major metabolite of ochratoxin A in all species examined is ochratoxin *alpha*, formed by hydrolysis of the peptide bond. Ochratoxin *alpha* and minor hydroxylated metabolites that have been identified are all reported to be less toxic than ochratoxin A itself. Ochratoxin A is excreted in urine and faeces, and the relative contribution of each of these routes in different species is influenced by the extent of the

enterohepatic recirculation of ochratoxin A and its binding to serum protein. These factors are also important in the determination of the serum half-life of ochratoxin A, which varies widely among species. It has a long half-life in some non-ruminant mammals (e.g. 1–1.5 days in mice, 2–5 days in rats, 3–5 days in pigs, 21 days in one macaque and 35 days in a human volunteer).

Additional information published since the Committee's previous evaluation is described below.

The metabolism of ochratoxin A was studied in cultured rat and human primary hepatocytes incubated with non-cytotoxic concentrations of [^{3}H]ochratoxin A ranging from 10^7 to 10^5 mol/l for 8 h. In rat hepatocytes, ochratoxin A was metabolized to small amounts of three products. In addition to 4-hydroxy-ochratoxin A, which is a known product of ochratoxin A biotransformation, two novel metabolites were detected and tentatively identified as hexose and pentose conjugates of ochratoxin A. In vitro induction with 3-methylcholanthrene increased the formation of 4-hydroxy-ochratoxin A but did not alter the formation of the conjugated metabolites (Gross-Steinmeyer et al., 2002).

A study was performed to investigate the biotransformation and toxicokinetics of orally administered ochratoxin A in rats. Groups of 18 male and 18 female F344 rats were given ochratoxin A at a single oral gavage dose of 0.5 mg/kg bw, in corn oil. Groups of three animals were sacrificed at intervals between 24 and 1344 h after ochratoxin A administration, and concentrations of ochratoxin A and its metabolites in urine, faeces, blood, liver and kidney were determined. There was efficient absorption of ochratoxin A from the gastrointestinal tract and only slow elimination and little biotransformation. Recovery of unchanged ochratoxin A in urine as a percentage of the dose was only 2.1% in males and 5.2% in females within 96 h. The majority of the dose was absorbed and still present in the systemic circulation or tissues at 96 h. In faeces, 5.5% and 1.5% of the dose were recovered in males and females, respectively, within 96 h. The major metabolite detected was ochratoxin *alpha*. Low concentrations of ochratoxin A–glucosides (pentose and hexose conjugates) were also reported for the first time in urine. The maximal blood levels of ochratoxin A were observed between 24 and 48 h after administration and were approximately 4.6 µmol/l in males and 6.0 µmol/l in females. Elimination of ochratoxin A from blood followed first-order kinetics, with a half-life of approximately 230 h. In liver of both male and female rats, concentrations of ochratoxin A were less than 12 pmol/g tissue, with a maximum at 24 h after administration. In contrast, ochratoxin A accumulated in the kidneys, reaching a concentration of 480 pmol/g tissue in males 24 h after administration of ochratoxin A. Ochratoxin *alpha* was not detected in liver and kidney tissue, and the concentrations in blood were low (10–15 nmol/l). The authors concluded that the high concentrations of ochratoxin A in kidneys of male rats may, in part, explain the organ- and sex-specific toxicity of ochratoxin A (Zepnik et al., 2003).

The EFSA opinion (European Food Safety Authority, 2006) discusses the hypothesis that specific transporters may be involved in the cellular uptake of ochratoxin A into the kidney, where it accumulates. It has been suggested that the marked differences in the relative sensitivity of individual species and sexes towards

ochratoxin A might be due to variations in the transport mechanisms and cellular uptake in renal cells. In vitro data with cell cultures indicate that ochratoxin A is a substrate for the family of organic anion transporter (OAT) proteins. In humans, the best characterized of these transporters, OAT1, encoded by the *SLC22A* gene family, has wide substrate specificity. The rodent equivalents of the human proteins are also encoded by members of the *SLC22A* gene family. Expression of OAT proteins in the kidney shows sex and species differences (Russel et al., 2002). Support for the hypothesis of an involvement of transporters in the observed sex and species differences in ochratoxin A–mediated toxicity is also to be found in the observations of Buist (2002) and Buist & Klaassen (2003), who demonstrated large sex-, age- and species-dependent variations in the expression levels of a number of OATs (Dietrich et al., 2005). Ochratoxin A is also a substrate for transporters of the adenosine triphosphate (ATP)–dependent transporter family, which excrete compounds out of cells (Schrickx et al., 2005). These transporters are assumed to play a role in the excretion of ochratoxin A–glucuronides by the kidneys.

Additional information on the toxicokinetics of ochratoxin A in primates is also available. Three female vervet monkeys, *Cercopithecus aethiops*, were each given a single intravenous dose of 0.8, 1.5 or 2 mg ochratoxin A/kg bw. Blood and urine samples were collected over a period of 21 days. The results suggested that the clearance of ochratoxin A from plasma followed a two-compartment model. The elimination half-life of ochratoxin A in the monkeys was 19–21 days, and the average total body clearance was 0.22 ml/h per kilogram body weight. The mean apparent volumes of distribution of the central compartment and the peripheral compartment were of similar value (59 ml/kg) (Stander et al., 2001).

The toxicokinetic profile of ochratoxin A was studied in one human volunteer following ingestion of 395 ng ^{3}H-labelled ochratoxin A (0.14 MBq). The data suggested a two-compartment open model. This two-compartment model consisted of a fast elimination and distribution phase (half-life about 20 h) followed by a slow elimination phase (renal clearance about 0.11 ml/min), with a calculated plasma half-life of 35 days. In addition, the intraindividual fluctuation of plasma levels of ochratoxin A was investigated in eight volunteers over a period of 2 months. The plasma concentrations determined ranged between 0.2 and 0.9 ng ochratoxin A/ml. The plasma levels in some individuals remained nearly constant over time, whereas others varied considerably (e.g. increase of 0.4 ng/ml within 3 days, decrease of 0.3 ng/ml within 5 days) during the observation period. The authors calculated a renal clearance varying between 0.093 and 0.109 ml/min (approximately 0.13 litres/day), indicating that even a non-regular exposure (consumption of contaminated foods once a week or once a month) can result in persistent blood levels (Studer-Rohr et al., 2000).

The major analyte in human blood serum was the parent compound, and only small concentrations of ochratoxin A metabolites and/or conjugates could be measured. In contrast, analysis of urine samples indicated that only about 50% of the radioactivity in the urine was parent ochratoxin A, suggesting the presence of ochratoxin A metabolites (particularly ochratoxin *alpha*) and/or ochratoxin A–glucuronic acid conjugates (Studer-Rohr et al., 2000).

2.2 Toxicological studies

2.2.1 Short-term studies of toxicity

The short-term toxicity of ochratoxin A has been extensively reviewed previously by this Committee (Annex 1, reference *153*) and by EFSA (European Food Safety Authority, 2006). In summary, the kidney is the major target organ for the adverse effects of ochratoxin A, and short-term toxicity studies in mice, rats, dogs and pigs have shown dose-dependent and time-dependent development of progressive nephropathy. There are significant sex and species differences in sensitivity of the kidney to the toxic effects of ochratoxin A. Most of the recent short-term studies on ochratoxin A–induced kidney toxicity have focused on the possible mechanism(s) for its toxic effects, and these are described below in section 2.2.5.

(a) Rats

Male F344/N rats were treated with ochratoxin A orally by gavage at doses of 0, 21, 70 or 210 µg/kg bw per day for 14, 28 or 90 days, 5 days/week. This treatment regimen corresponded to that used in the 2-year bioassay conducted by the United States National Toxicology Program (NTP) (National Toxicology Program, 1989), which established the renal carcinogenicity of low-dose ochratoxin A in rats. Cell proliferation in the renal cortex and the outer stripe of the outer medulla was determined. Histopathological examination showed renal changes in mid- and high-dose animals involving single cell death and prominent nuclear enlargement within the straight segment of the proximal tubules of the outer stripe of the outer medulla, from which ochratoxin A–induced tumours are known to arise. At doses of 70 and 210 µg/kg bw per day, there was a marked dose- and time-dependent increase in renal cell proliferation, extending from the medullary rays into the outer stripe of the outer medulla. No effects were evident in the kidneys of low-dose animals or in the liver. The no-observed-effect level (NOEL) in this study was 21 µg/kg bw per day, which correlates with the dose in the NTP 2-year bioassay that did not produce renal tumours. The apparent correlation between enhanced cell turnover and tumour formation induced by ochratoxin A indicates that stimulation of cell proliferation may play an important role in ochratoxin A carcinogenicity (Rached et al., 2007).

There have been several reports on ochratoxin A–induced apoptosis in the kidney. In rats given ochratoxin A intraperitoneally at doses of 0.25, 0.50 or 1.00 mg/kg bw, 3 times a week for 4 weeks, time- and dose-related increases in apoptosis in the kidneys were observed (Domijan et al., 2004).

Intraperitoneal administration of 120 µg ochratoxin A/kg bw per day to Wistar rats, for 10, 30 or 60 days, produced oxidative stress and dose/time-related apoptosis in both proximal and distal epithelial kidney cells. Ochratoxin A concentrations in the kidneys were proportional to the time of exposure and amounted to 547, 753 and 930 ng/g kidney tissue after 10, 30 and 60 days, respectively. Oxidative stress was evident from increased malondialdehyde formation in the kidney cells (Petrik et al., 2003).

Ochratoxin A was administered orally by gavage to groups of three male Fischer 344 rats at dose levels of 0, 0.25, 0.5, 1 or 2 mg/kg bw per day for 2 weeks. The typical pathological changes, including apoptosis, were seen in the kidneys at all dose levels, with a clear increase in severity with higher doses. A dose-dependent increase in the expression of proliferating cell nuclear antigen, indicative of cell proliferation, was observed in kidneys, but not in livers, of treated animals. The most prominent change in the composition of urine induced by ochratoxin A consisted of a major increase in the excretion of trimethylamine *N*-oxide. This pattern was said to be different from the typical changes observed with other proximal tubular toxins and suggests that a unique mechanism may be involved in ochratoxin A nephrotoxicity and carcinogenicity (Mally et al., 2005a).

(b) Rabbits

Ochratoxin A was fed in the diet to groups of four New Zealand White rabbits, starting at 6–8 weeks of age, at a concentration of 0 or 0.75 mg/kg of feed for 60 days, as part of an investigation into the effects of co-administration of ochratoxin A with another mycotoxin, citrinin. Ultrastructural changes in the epithelial cells of the proximal convoluted tubule in the kidney were evaluated. Degenerative and necrotic changes were observed, especially in the mitochondria, together with loss of the brush border, atrophy of microvilli, cytoplasmic vacuolation with loss of organelles, degenerating nucleus and loss of nucleolus (Kumar et al., 2007).

(c) Pigs

As described in the previous evaluation of ochratoxin A by this Committee (Annex 1, reference *153*), the pig appears to be the most sensitive species with respect to the nephrotoxic effects of ochratoxin A, and the LOEL in pigs for renal effects is the basis of the PTWI.

Groups of three male and three female pigs were exposed to diets contaminated with strains of *Aspergillus ochraceus* producing ochratoxin A and penicillic acid and containing 90, 130 or 180 µg ochratoxin A/kg of diet. Two pigs from each group were examined after 3 months. Microscopic lesions, as well as changes in various haematological and biochemical parameters, were observed in all groups. For an additional 2 months, the ochratoxin A concentrations in the diets were raised to 130, 305 or 790 µg/kg. Histological examination (two pigs per group) at the end of the exposure period showed degenerative changes affecting the epithelial cells of the proximal tubules, which predominated at the initial stage, and proliferative changes in the interstitium, which predominated at the later stage of the disease (Stoev et al., 2001). Subsequently, Stoev et al. (2002) reported only

mild nephropathy in three male and three female pigs given a diet containing ochratoxin A at a concentration of 800 µg/kg for 1 year. Histological examination showed two types of changes: degenerative changes affecting epithelial cells in some proximal tubules of pigs after 6 months, and proliferative changes in the interstitium, which predominated after 1 year of exposure to ochratoxin A.

2.2.2 Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenicity of ochratoxin A have been extensively reviewed previously by this Committee (Annex 1, reference *153*) and by EFSA (European Food Safety Authority, 2006). Ochratoxin A produces renal tumours in mice and rats, and, as is the case for short-term toxicity to the kidney, there are marked sex and species differences. Male animals are more sensitive than females, and rats are considerably more sensitive than mice. The Committee previously noted that the long-term effects in rats were preceded by evidence of renal toxicity in 16-day and 13-week studies and that it was unclear whether the malignancy and aggressive nature of the tumours were a secure indication that the mechanism of induction is via deoxyribonucleic acid (DNA) reactivity (Annex 1, reference *153*).

The several hypotheses on the mode of action of ochratoxin A as a carcinogen and the related evidence have been discussed elsewhere (see, for example, reviews by Fink-Gremmels, 2005; Walker & Larsen, 2005; Rached et al., 2007). These hypotheses will be discussed in section 9, in light of the additional evidence obtained since the Committee's previous review in 2001.

The key features of the carcinogenicity bioassays in mice and rats conducted by the NTP, reviewed previously by this Committee, together with information from these and other related NTP studies concerning the occurrence of karyomegaly as an early pathological indicator of renal toxicity, are summarized below. It should be noted that for male rats, the LOELs and NOELs for karyomegaly are similar to those for tumour formation (Table 1).

Table 1. LOELs and NOELs for karyomegaly and carcinogenicity of ochratoxin A in male mice and rats[a]

Species	Effect	Study duration	LOEL (µg/kg bw per day)	NOEL (µg/kg bw per day)
Mouse (male)[b]	Kidney tumours	2 years	4400	130
Rat (male)[c]	Karyomegaly of proximal tubule cells	90 days	62.5	Not established
		9 and 15 months	70	21
	Kidney tumours	2 years	70	21

[a] From National Toxicology Program (1989).
[b] Ochratoxin A administered in the diet.
[c] Ochratoxin A administered by gavage 5 days/week.

Some new data on the carcinogenicity of ochratoxin A have also been presented in the final report of the Ochratoxin A Risk Assessment project, funded by the European Commission (2004). Fischer 344 rats were given ochratoxin A in the feed at a concentration giving intakes equivalent to approximately 300 µg/kg bw per day. A statistical comparison of the total carcinoma incidence from this exposure with that reported in the NTP Fischer 344 rat study (National Toxicology Program, 1989), in which gavage administration had been used, suggested that ochratoxin A is significantly less potent when given in feed than when given by oral gavage. However, the Committee noted that EFSA considered it unsuitable as a basis for a quantitative risk assessment because there was only one dose level in the feed (European Food Safety Authority, 2006), and further details of the study were lacking (Mantle et al., 2005).

In order to provide additional information for the risk assessment, the Committee performed benchmark dose (BMD) modelling using the carcinogenicity data on ochratoxin A from the rat bioassay performed by the NTP (National Toxicology Program, 1989). The occurrence of combined adenomas and carcinomas in the kidneys of the male rats (Table 2), as the most sensitive sex and species for kidney carcinogenicity of ochratoxin A, was considered the most appropriate data for modelling.

The BMD approach has been put forward as an alternative to the no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) approach for health effects because it provides a more quantitative alternative point of departure for the first step in the dose–response assessment (International Programme on Chemical Safety, in press). The BMD approach is based on a mathematical model being fitted to the experimental data within the observable range and estimates the dose that causes a low but measurable response (the benchmark response) typically chosen at a 5% or 10% incidence above the control. The BMD lower limit (BMDL) refers to the corresponding lower limit of a one-sided 95% confidence interval on the BMD. Using the lower bound takes into account the uncertainty inherent in a given study and assures (with 95% confidence) that the chosen benchmark response is not exceeded.

Table 2. Renal tumours and karyomegaly in male rats exposed to ochratoxin A[a]

Ochratoxin A dose (µg/kg bw per day)[b]	Adenomas	Carcinomas	Adenomas and carcinomas	Karyomegaly
0	1/50	0/50	1/50	0/50
21	1/50	0/50	1/50	1/50
70	6/51	16/50	20/51	51/51
210	10/50	30/50	36/50	50/50

[a] From National Toxicology Program (1989).
[b] Administered by gavage on 5 days/week for 2 years.

The United States Environmental Protection Agency BMD software version 1.4.1 was used (United States Environmental Protection Agency, 2007) for modelling the kidney tumour dose–response in the male rats. For carcinogenicity data, a number of models are available in the BMD software, and model fitting, determination of goodness of fit and comparing models to decide which one to use for obtaining the BMDL are outlined. The following dose–response models were fitted to the dose–incidence data:

- gamma multihit model;
- log-logistic model;
- multistage model;
- probit model;
- log probit model;
- quantal linear model;
- Weibull model.

The BMD_{10} and $BMDL_{10}$ values, for an extra 10% increase in incidence of tumours compared with the background incidence in controls, were estimated by performing 250 iterations.

The acceptability of a model can be based on several criteria. Some of the models are nested models (i.e. they are related to each other such that by leaving out a parameter, one model reduces to the other; this holds for the one-, two- and three-stage models). The fit should not be significantly worse (using the likelihood ratio test) than the fit provided by the "full" model. The full model is the model that does not assume any dose–response function (its parameters are simply the frequencies per dose level) (Filipsson et al., 2003).

While the likelihood ratio test can be applied only to nested models, the Akaike information criterion (AIC) (Akaike, 1974; Bozdogan, 1987) has been proposed as an approximate criterion for comparing the fits of non-nested models (Filipsson et al., 2003).

In addition, the BMD software provides statistics for the goodness of the fit. The lower the chi-square value, the better the fit, and the calculated *p*-value should be significantly larger than 0.1, which in this case was chosen to represent a rejection level (Filipsson et al., 2003).

The BMD_{10} and $BMDL_{10}$ values, as well as the associated statistics, for the models used are presented in Table 3.

The calculated BMD_{10} values ranged from 18 to 33 µg ochratoxin A/kg bw per day, with the most reliable values being around 30 µg ochratoxin A/kg bw per day. The $BMDL_{10}$ values ranged from the lowest value of 15 µg/kg bw per day up to 25 µg/kg bw per day for the model providing the best fit. Thus, the $BMDL_{10}$ does not provide a lower point of departure, compared with the LOEL of 8 µg/kg bw per day for minimal renal toxicity changes in the pig, for establishing the PTWI.

Table 3. BMD_{10} and $BMDL_{10}$ calculations based on total kidney tumour incidences in male F344 rats from the NTP study[a]

Model	Log (likelihood)	*p*-value	AIC	Chi-square	*P*-value	Accept	BMD_{10} (μg/kg bw per day[b])	$BMDL_{10}$ (μg/kg bw per day[b])
Full model	–71.61							
Gamma multi-hit	–76.36	0.02	158.7	4.91	0.03	??	30	18
Log-logistic	–75.57	0.05	157.1	3.46	0.06	Yes??	32	21
Multistage	–77.29	0.01	160.6	5.96	0.01	??	24	15
Log-probit	–75.05	0.09	156.1	2.64	0.1	Yes	33	25
Quantal-linear	–77.74	0.02	159.5	5.99	0.05	??	18	15
Weibull	–76.68	0.01	159.4	5.27	0.02	??	28	17
Reduced model	–120.77	<0.001						

[a] From National Toxicology Program (1989).
[b] Ochratoxin A administered by gavage 5 days/week for 2 years.

2.2.3 Genotoxicity

Numerous in vitro and in vivo genotoxicity studies have been conducted on ochratoxin A, most of which were described previously by this Committee (Annex 1, reference *153*). Further studies have been conducted since then to try to clarify the mode of action of ochratoxin A as a carcinogen. Since genotoxicity data are a key element in the present evaluation, a summary of all the relevant data is presented in Table 4.

(a) DNA adducts

The question of whether ochratoxin A can form DNA adducts in the kidney has been intensively studied because of its potential relevance to a genotoxic mode of action for ochratoxin A as a kidney carcinogen. The information on the earlier and more recent studies has been reviewed by several groups (Annex 1, reference *153*; Mally & Dekant, 2005; Pfohl-Leszkowicz & Castegnaro, 2005; Turesky, 2005; European Food Safety Authority, 2006; Pfohl-Leszkowicz & Manderville, 2007).

At the time of the Committee's previous evaluation (Annex 1, reference *153*), almost all the available studies in which DNA adducts were detected by ^{32}P-postlabelling after exposure to ochratoxin A were from one laboratory, and all showed positive results, including in rat and mouse kidney DNA (Pfohl-Leszkowicz et al., 1991, 1993; Grosse et al., 1995, 1997; Castegnaro et al., 1998; Pfohl-Leszkowicz et al., 1998). However, the Committee pointed out that the nonspecific postlabelling technique used may have resulted in adducts that did not

Table 4. Summary of results of genotoxicity tests on ochratoxin A

Test system	Test object	Concentration	Results	Reference
In vitro				
Bacterial assays				
Reverse mutation	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	0.4–400 µg/plate	Negative (highly variable TA100 controls, not tested to cytotoxicity)	Kuczuk et al. (1978); Wehner et al. (1978)
Reverse mutation	*S. typhimurium* TA1538	0.1–500 µg/plate (mixture of ochratoxin A:ochratoxin B)	Positive >100 µg/plate	Kuczuk et al. (1978)
Reverse mutation	*S. typhimurium* TA100 and TA1538	~200 µg/plate	Negative with mouse and rat liver activation	Bartsch et al. (1980)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	50–600 µg/plate	Negative	Bendele et al. (1985)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538, G46, G3076 and D3052	0.1–100 µg/ml	Negative	Bendele et al. (1985)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100 and TA1535	1–100 µg/plate	Negative with hamster or rat liver activation	National Toxicology Program (1989)
Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538		Positive after activation by medium derived from hepatocytes exposed to ochratoxin A	Hennig et al. (1991)
Reverse mutation	*S. typhimurium* TA98, TA1535 and TA1538 (TA100 and TA102 also tested in preliminary study)	0–1200 µg/plate	Positive in all 3 strains after activation by mouse kidney microsomes, but not after mouse liver microsomes (TA100, TA102 negative)	Obrecht-Pflumio et al. (1999)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
Reverse mutation	*S. typhimurium* TA100 and TA2638	0–200 µg/plate	Negative after activation with rat liver and kidney microsomes	Zepnik et al. (2001)
Reverse mutation	*S. typhimurium* TA98 and TA100	2.5–50 µg/plate	Negative with HepG2-derived enzyme homogenate (S9 mix)	Ehrlich et al. (2002)
Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA104, TA1535 and TA1538	0.01–500 µmol/l	Negative with and without rat liver S9 mix	Föllmann & Lucas (2003)
Gene mutation	*Saccharomyces cerevisiae* D3	75, 200 µg/plate	Negative	Kuczuk et al. (1978)
Gene mutation	*Bacillus subtilis* rec	20–100 µg/disc	Negative	Ueno & Kubota (1976)
DNA repair	*Escherichia coli*, SOS assay	1–2 mg/100 µl	Negative	Auffray & Boutibonnes (1986); Reiss (1986)
DNA repair	*E. coli* WP2	Gradient plate, not stated	Negative	Bendele et al. (1985)
Mammalian cells				
Gene mutation	C3H mouse mammary cells	5–10 µg/ml	Negative (10 µg/ml cytotoxic)	Umeda et al. (1977)
Forward gene mutation	Mouse lymphoma cells, *tk* locus	0.1–13 µg/ml	Negative (>12 µg/ml cytotoxic)	Bendele et al. (1985)
Forward gene mutation	Chinese hamster V79 fibroblasts, *HPRT*	0.1–100 µmol/l	Negative with and without rat liver S9 mix	Föllmann & Lucas (2003)
Gene mutation (*lacZ* on shuttle vector)	NIH/3T3 cells transfected with human cytochrome P450	25 µg/ml	Positive	De Groene et al. (1996)
UDS	ACI rat primary hepatocytes	0.4, 4 µg/ml	Weakly positive at 0.4, cytotoxic at 4 µg/ml	Mori et al. (1984)
UDS	C3H mouse primary hepatocytes	4, 40 µg/ml	Weakly positive at 4, cytotoxic at 40 µg/ml	Mori et al. (1984)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
UDS	Fischer 344 rat primary hepatocytes	0.000 025–500 µg/ml (2 lots tested at 15 doses)	Negative (>0.05 µg/ml cytotoxic)	Bendele et al. (1985)
UDS	Rat hepatocytes; porcine urinary bladder epithelial cells	250 nmol/l–1 µmol/l	Positive	Dorrenhaus & Föllmann (1997)
UDS	Cultured human urothelial cells	0.005–0.05 µmol/l	Positive	Flieger et al. (1998)
UDS	Primary human urothelial cells	10–2000 nmol/l	Positive	Dorrenhaus et al. (2000)
DNA strand break, alkaline elution	CHO cells; rat fibroblasts	200 µg/ml	Positive (1.2 strand breaks/109 Da)	Stetina & Votava (1986)
DNA damage	Mouse spleen, phytohaemagglutinin-stimulated	1–10 µg/ml	Positive (concentration-related)	Creppy et al. (1985)
DNA damage, comet assay	Human hepatoma-derived cell line, HepG2	5–30 µg/ml	Positive	Ehrlich et al. (2002)
DNA damage, comet assay	MDCK cells		Positive (concentration-related with and without rat liver S9 mix)	Lebrun & Föllmann (2002)
DNA damage, comet assay	Primary cultured human urothelial cells	100 µmol/l	Variable results, DNA damage was associated with certain polymorphisms of GST enzymes	Lebrun et al. (2006)
DNA damage, comet assay	Mouse fibroblast NIH/3T3 cells transfected with human cytochrome P450 co-expressing human oxidoreductase	10–200 µmol/l	Negative in CYP3A4-expressing cells, positive in CYP2C9-expressing cells	Simaro Doorten et al. (2006)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
DNA damage, comet assay	Human kidney proximal tubular cell line, HK-2	50–600 µmol/l	Negative at non-cytotoxic concentrations (with or without rat liver S9 mix) at 3 h; positive at cytotoxic concentrations (≥400 µmol/l) at 6 h and at all concentrations with Fpg and Endo III (for detection of oxidative damage)	Arbillaga et al. (2007a, 2007b)
SCE	Human peripheral blood lymphocytes	5–10 µg/ml	Negative (mitotic inhibition at 10 µg/ml)	Cooray (1984)
SCE	CHO cells, 26 h with ochratoxin A	0.5–5 µg/ml	Negative	National Toxicology Program (1989)
SCE	CHO cells, 2 h with ochratoxin A	5–160 µg/ml	Positive (frequency 37% above control, weak dose–response relationship)	National Toxicology Program (1989)
SCE	Human lymphocytes		Positive	Hennig et al. (1991)
SCE	Bovine lymphocytes	0.1–2 µmol/l	Positive (in the presence of reduced cell proliferation and viability and increased apoptosis)	Lioi et al. (2004)
Chromosomal aberration	CHO cells, 8–10 h with ochratoxin A	30–160 µg/ml	Negative	National Toxicology
	2 h with ochratoxin	100–300 µg/ml	Negative	Program (1989)
Chromosomal aberration	Human lymphocytes, 48 h with ochratoxin A	4.5 µg/ml	Positive (4.5- to 5-fold increase)	Manolova et al. (1990)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
Chromosomal aberration	Bovine lymphocytes	0.1–2 µmol/l	Positive (in the presence of reduced cell proliferation and viability and increased apoptosis)	Lioi et al. (2004)
Micronucleus formation	Ovine seminal vesicle cell cultures	12–30 µmol/l	Positive[a]	Degen et al. (1997)
Micronucleus formation	Syrian hamster embryo fibroblasts	5–20 µmol/l	Positive[b]	Dopp et al. (1999)
Micronucleus formation	Human hepatoma-derived cell line, HepG2	5–50 µg/ml	Positive (via chromosome breaking effects)	Ehrlich et al. (2002)
In vivo				
Chromosomal aberration	Mouse bone marrow and sperm cells	1 µg/kg bw per day in diet, 15 and 45 days	Positive (ameliorated by 10 mg ascorbic acid/kg bw)	Bose & Sinha (1994)
Chromosomal aberration	Mouse	1 µg/kg bw per day in diet, 14 days	Positive (ameliorated by 130 IU vitamin A/kg bw)	Kumari & Sinha (1994)
Chromosomal aberration	Rat, cultured spleen lymphocytes from animals treated in vivo	250–2000 µg/kg bw by gavage 5 days/week for 2 weeks	Slight increases in aberrations (mainly deletions), but not statistically significant	Mally et al. (2005b)
SCE	Chinese hamster bone marrow	25–400 mg/kg bw by gavage	Negative (>100 mg/kg bw cytotoxic)	Bendele et al. (1985)
DNA damage (single-strand breaks)	BALB/c mouse Spleen 4,16 and 24 h after treatment	2500 µg/kg bw intraperitoneally	Positive (maximum response at 24 h)	Creppy et al. (1985)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
	Kidney 24 and 48 h after treatment		Positive (maximum response at 24 h)	
	Liver 24, 48 and 72 h after treatment		Positive (maximum response at 48 h; recovery at 72 h)	
DNA damage	Wistar rat kidney and liver	290 µg/kg bw by gavage every 48 h for 6 or 12 weeks	Positive, no recovery between treatments	Kane et al. (1986)
DNA damage, comet assay	Rat liver, kidney and spleen	250–2000 µg/kg bw by gavage 5 days/ week for 2 weeks	Positive, enhanced by Fpg glycosylase (converts oxidative DNA damage into strand breaks)	Mally et al. (2005b)
DNA damage, comet assay	Rat kidney	500 µg/kg bw per day intraperitoneally for 7, 14 or 21 days	Positive (extent of DNA damage correlated with increasing plasma and kidney ochratoxin A levels over time)	Zeljezic et al. (2006)

CHO, Chinese hamster ovary; CYP, cytochrome P450; Endo III, endonuclease III; Fpg, formamido-pyrimidine-DNA-glycosylase; GST, glutathione *S*-transferase; IU, international units; MDCK, Madin-Darby canine kidney; S9, 9000 × *g* supernatant from rat liver; SCE, sister chromatid exchange; UDS, unscheduled DNA synthesis.

[a] No inhibition by indomethacin, suggesting absence of activation by prostaglandin H synthase.

[b] Clastogenic effects due to changes in intracellular calcium.

contain an ochratoxin A or ochratoxin A metabolite moiety and that at least some of the adducts might have been due to ochratoxin A–induced cytotoxic effects generating reactive oxygen species (ROS). Other studies in vitro, using purified DNA and mononucleotides incubated with kidney or liver microsomes from mouse and rabbit, ochratoxin A and either reduced nicotinamide adenine dinucleotide phosphate (NADPH) or arachidonic acid as cofactors, indicated that oxidative damage to DNA might not be the only source of the presumed adducts (Obrecht-Pflumio & Dirheimer, 2000).

In contrast to these results with ^{32}P-postlabelling methods, others reported that the level of covalent binding of [^{3}H]ochratoxin A or its metabolites to DNA was below the limit of detection of scintillation counting in rodent kidney and liver in vivo or in rat or human hepatocytes in vitro (Rasonyi, 1995; Schlatter et al., 1996; Gautier et al., 2001; Gross-Steinmeyer et al., 2002). However, Gautier et al. (2001) did report a small increase in DNA adduct levels in male Fischer 344 rats treated orally with [^{3}H]ochratoxin A compared with controls, using the ^{32}P-postlabelling assay, and suggested that these may be due to products derived from ochratoxin A–mediated cytotoxicity.

In the more recent studies, using the ^{32}P-postlabelling assay, with nuclease P1-enrichment, thin layer chromatography (TLC) analysis showed that several spots could be detected in chromatograms of the DNA fraction of liver and kidneys of rats and mice chronically exposed to high levels of ochratoxin A (0.4–2.5 mg/kg bw per day) for 2 years. Adduct levels were almost undetectable at 0.6 mg/kg bw, were low at 1.2 mg/kg bw but were quantifiable at 2.5 mg/kg bw following 48 or 72 h of incubation. The largest number of TLC spots was found in chromatograms of DNA from male DA rats, a strain highly susceptible to ochratoxin A–induced kidney carcinogenesis, with fewer spots in females and in Lewis rats. The higher relative adduct level in male mouse kidney as compared with the liver and the female kidneys is consistent with the observation that male mice are more susceptible than females to renal carcinogenesis (Faucet et al., 2004; Manderville, 2005; Pfohl-Leszkowicz & Castegnaro, 2005). As pointed out by EFSA (European Food Safety Authority, 2006), the chemical structure of the spots visible in the TLC chromatograms was not identified, and hence there was no indication that the spots represent genuine ochratoxin A–DNA adducts.

The possible role of lipid peroxidation (LPO) in the formation of DNA adducts has been considered. In studies cited by EFSA (European Food Safety Authority, 2006) involving substances other than ochratoxin A that generate LPO products, several exocyclic DNA adducts derived from LPO products have been identified, such as ethenobases, formed by reaction of *trans*-4-hydroxy-2-nonenal with DNA. Another major DNA lesion associated with LPO is the adduct formation by the reaction of malondialdehyde with guanine. As EFSA has pointed out (European Food Safety Authority, 2006), it therefore cannot be excluded that the DNA adducts shown as spots observed in studies with ochratoxin A using the ^{32}P-postlabelling technique are due to these adducts and hence are not related to ochratoxin A itself, but instead are formed via an indirect mechanism. This hypothesis is supported by the observation that treatment of animals with antioxidants prior to ochratoxin A exposure significantly reduced the number and intensity of DNA fragments formed (Pfohl-Leszkowicz et al., 2002). However, one group has used the ^{32}P-postlabelling assay to search for LPO-related adducts in the kidneys of rats treated with ochratoxin A at doses of 0, 0.25, 0.5, 1 or 2 mg/kg bw for 5 days/week for 2 weeks but could not detect any, nor was there any other evidence of LPO (Mally et al., 2005a).

It has also been postulated that DNA adducts might originate from a reaction between the hydroquinone derived from ochratoxin A and cellular DNA. The presence of hydroquinone has been demonstrated in rat urine (Mally et al., 2004).

To investigate this hypothesis, DNA adduct formation was studied in vitro using [^{3}H]ochratoxin A as substrate and horseradish peroxidase and prostaglandin H synthase as activation system to facilitate the formation of the hydroquinone. Using liquid scintillation counting, no DNA adducts were found (Gautier et al., 2001).

It has, however, been shown that C8 ochratoxin A-3′-monophosphate-deoxyguanosine (OTA-3′-dGMP) adducts can be generated by photoirradiation (Faucet et al., 2004). It was hypothesized that, in vivo, an ochratoxin A phenoxy radical, ochratoxin quinone (OTQ), is formed by oxidative dechlorination, and the radical could interact with DNA to form such an adduct. OTQ could be reduced to ochratoxin hydroquinone (OTHQ) and OTHQ autoxidized back to OTQ (Dai et al., 2004). While the chemical structure of ochratoxin A–DNA adducts formed in vivo has not been completely characterized, it has been suggested that they represent C-C8 and O-C8 OTA-3′-dGMP, because these synthetic adducts appear to co-chromatograph with adducts found using the ^{32}P-postlabelling technique in kidneys of pig following subacute exposure (0.20 mg/kg bw) and in rats after chronic exposure (3 times weekly, 2 years; total dose 100 mg/kg bw) (Faucet et al., 2004; Pfohl-Leszkowicz & Castegnaro, 2005). However, as EFSA has pointed out (European Food Safety Authority, 2006), the co-chromatography was demonstrated with only one set of chromatographic conditions and thus remains to be confirmed.

Further work by the same group, using the ^{32}P-postlabelling technique, has shown that a quinone/hydroquinone redox couple derived from ochratoxin A may play a role in ochratoxin A–mediated genotoxicity. The adduction properties of ochratoxin A and hydroquinone were compared in salmon sperm DNA, in the absence and presence of pig kidney microsomes as the metabolic activation system. In the absence of metabolic activation, ochratoxin A did not generate DNA adducts, but hydroquinone did generate DNA adducts. The authors ascribed these adducts to covalent DNA adduction by quinone, generated through autoxidation of the hydroquinone precursor, OTHQ. The quinone-mediated adduct spots noted with hydroquinone were also observed when ochratoxin A was incubated with pig kidney microsomes and NADPH, suggesting that ochratoxin A undergoes oxidative activation to quinone by cytochrome P450 or enzymes with peroxidase activity. Adduct formation was also compared in human kidney and bronchial epithelial (WI26) cell lines, in the presence of metabolic activation. DNA adducts were formed in a dose- and time-dependent manner both with ochratoxin A and with hydroquinone, but the rate of adduct formation was faster with hydroquinone, presumably reflecting a more ready formation of quinone from hydroquinone (Tozlovanu et al., 2006). However, quinone-mediated adducts do not co-migrate with C-C8 and O-C8 OTA-3′-dGMP standard adducts, suggesting that the quinone electrophile is not responsible for the formation of C8-deoxyguanosine (C8-dG) adducts that are produced by photoactivation of ochratoxin A. Moreover, DNA adducts deriving from hydroquinone are mainly formed after biotransformation of ochratoxin A by CYP2C9 enzyme (with epoxygenase activity), whereas C8-dG adducts are formed after biotransformation by 5-lipoxygenase, which is a microsomal glutathione (GSH) enzyme (Pfohl-Leszkowicz & Manderville, 2007).

A study on the possible involvement of biotransformation of ochratoxin A via the GSH pathway in production of DNA adducts has been conducted in the opossum kidney (OK) cell line derived from the proximal tubule of American

opossum. The cells were treated with ochratoxin A alone or in the presence of agents such as 2-mercaptoethane sulfonate and *N*-acetyl-L-cysteine, which reduce oxidative stress by increasing free thiols in the kidney, buthione sulfoximine, an inhibitor of GSH synthase, and α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid, an inhibitor of gamma glutamyl transpeptidase. None of these agents reduced ochratoxin A–induced cytotoxicity in the OK cells, and all enhanced ochratoxin A biotransformation. Both the ochratoxin A metabolic profiles produced and the nature of the DNA adducts formed during incubation with these agents differed qualitatively and quantitatively according to the agent. The findings suggested that the mechanism by which ochratoxin A results in DNA adduct formation is closely related to its biotransformation by a pathway involving quinone derivatives and GSH conjugation (Faucet-Marquis et al., 2006).

Other experiments have also aimed to identify the chemical structure of potential ochratoxin A adducts. In in vitro studies, incubation of ochratoxin A with DNA or dG in the presence of various activation systems did not reveal any adducts, neither by ^{32}P-postlabelling nor by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Subsequently, DNA was isolated from the livers and kidneys of male Fischer rats treated with a single dose of [^{14}C]ochratoxin A (0.5 mg/kg bw) and analysed by ^{14}C-accelerated mass spectrometry. No significant difference between control group and treated group could be observed in ^{14}C activity in isolated DNA from treated animals, and no specific ochratoxin A–DNA adducts were detected with ^{14}C-accelerated mass spectrometry (Mally et al., 2004). The EFSA opinion pointed out that a concern in the interpretation of these results is that DNA adducts may have been repaired, as DNA was isolated 72 h after a single treatment with a relatively low dose, which is in contrast to other studies, in which DNA was isolated within 24 h after exposure to ochratoxin A (European Food Safety Authority, 2006).

In an effort to improve on the previous methodology, the same group has developed a stable isotope dilution LC-MS/MS method to analyse for ochratoxin A–dG adduct (dGuoOTA) in kidney DNA. DNA was isolated from the kidneys of male Fischer 344 rats treated orally with 210 µg ochratoxin A/kg bw per day for 90 days. The dose selected is known to cause renal tumours if given to rats chronically. Despite the sensitivity of the method (limit of detection [LOD] was calculated to be as low as 3.5 dGuoOTA/10^9 nucleotides), dGuoOTA was not detected in the kidney DNA of treated animals (Delatour et al., in press).

(b) DNA damage and repair

As summarized previously by this Committee, there was no evidence of DNA repair as a result of possible DNA damage in bacteria, whereas DNA single-strand breaks were consistently induced in cultured mammalian cells and were also observed in vivo in spleen, liver and kidney cells of mice after intraperitoneal injection of ochratoxin A. DNA repair, manifested as unscheduled DNA synthesis, was observed in most studies with primary cultures of rat and mouse hepatocytes, porcine epithelial cells from bladder and human urothelial cells (Annex 1, reference *153*).

In the more recent studies, there is further evidence of DNA damage, as assessed in the comet assay both in vitro, in mouse fibroblasts, rat liver, dog kidney, human kidney and human urothelial cells (Ehrlich et al., 2002; Lebrun & Föllmann, 2002; Lebrun et al., 2006; Simaro Doorten et al., 2006; Arbillaga et al., 2007a, 2007b), and in vivo, in rat liver, kidney and spleen (Mally et al., 2005b; Zeljezic et al., 2006). In some of these studies, the amount of DNA damage was enhanced by agents that highlight oxidative damage.

(c) Gene mutation

As summarized previously by this Committee, most tests for induction of gene mutations in bacteria showed no effect of exposure to ochratoxin A, whereas two studies showed positive results (Annex 1, reference *153*). Two recent studies on gene mutation in bacteria were negative (Ehrlich et al., 2002; Föllmann & Lucas, 2003). The Committee noted that in none of the bacterial studies was ochratoxin A tested up to cytotoxic concentrations or alternatively up to the highest concentration (5 mg/plate) recommended in current test guidelines. The Committee also noted that in those studies utilizing *Salmonella* strains TA102, TA104 or TA2638 or *Escherichia coli* strain WP2, all of which are known to be sensitive to oxidative stress, the results were negative.

In mammalian cells, gene mutations were not induced in three studies, whereas positive results were observed in one (Annex 1, reference *153*; Föllmann & Lucas, 2003).

(d) Chromosomal aberration

As summarized previously by this Committee, sister chromatid exchange was induced in two of four studies in vitro but not in a single study in vivo after gavage of a range of doses that included cytotoxic doses (Annex 1, reference *153*). A more recent in vitro study was positive (Lioi et al., 2004).

As summarized previously by this Committee, in tests for chromosomal aberrations in vitro, there was one positive and one negative study; in tests for micronucleus formation in vitro, there were two positive results. Two more recent in vitro tests on chromosomal aberration (Lioi et al., 2004) and micronucleus formation (Ehrlich et al., 2002) were also positive. In vivo, as summarized previously by this Committee, chromosomal aberrations were induced in mice (Annex 1, reference *153*). In a more recent study, results in rats were negative (Mally et al., 2005b).

2.2.4 Reproductive and developmental toxicity

No adequate studies on the reproductive toxicity of ochratoxin A were available for review. Several studies on developmental toxicity, showing that ochratoxin A can cross the placenta and that it is embryotoxic and teratogenic in rats and mice, were described previously by this Committee (Annex 1, reference *153*). Studies published since 2001 are summarized below.

(a) Rats

Groups of 10 Wistar rats were given ochratoxin A by oral gavage at doses of 0, 0.125, 0.25, 0.50 or 0.75 mg/kg bw per day on days 6–15 of gestation. Ochratoxin A caused increases in embryonic and fetal death at 0.25 mg/kg bw per day and above, as seen by increased incidences of dams with complete resorption/abortion and dams with some resorptions and a reduced number of live fetuses. These effects were dose related and reached statistical significance at the highest dose. Fetal weight and crown–rump length were also reduced in a dose-dependent way, and the reductions were statistically significant at the highest dose. Gross malformations and skeletal and visceral anomalies were all increased at all doses, in a dose-dependent manner, and the increases were statistically significant at 0.5 and 0.75 mg/kg bw per day. Gross malformations included exencephaly, incomplete closure of skull, micrognathia, micromelia, curly tail, scoliosis and small hind portion. Skeletal anomalies included incomplete ossification in a number of bones and fused, wavy or branched ribs. Visceral defects included hydrocephalus, microphthalmia, dilated renal pelvis, hydronephrosis and cryptorchid testis. Histological examination of liver, kidney, brain and eyes in subsets of fetuses undergoing visceral examination showed an increased incidence of lesions in those from dams given 0.25 mg/kg bw per day and above. The lesions included oedema, fibrosis and epithelial degeneration in the kidney, hepatocyte degeneration, bile duct proliferation, hypoplasia of the cerebellum and defects of the lens and retina (Wangikar et al., 2004a, 2004b). The same group has shown that the minimum teratogenic dose of ochratoxin A in Wistar rats, when given as a single oral gavage dose on one of days 6–15 of gestation, is 2.75 mg/kg bw per day. The most sensitive days for inducing teratogenicity were day 6 and day 7 of gestation (day of finding sperm in vaginal smear designated day 0) (Patil et al., 2006).

(b) Rabbits

Groups of five New Zealand White rabbits were given ochratoxin A by oral gavage at doses of 0, 0.025, 0.05 or 0.10 mg/kg bw per day on days 6–18 of gestation. In the highest dose group, there were significant decreases in fetal weight and number of live fetuses. There was an increased incidence of fetuses with malformations or anomalies (knuckling of fetlock, rudimentary tail or agenesis of tail, wavy ribs, hydrocephalus, microphthalmia, agenesis of kidney, reduced ossification of the skull and failed ossification in the caudal vertebrae). Histological examination of liver, kidney, brain and eyes showed a dose-related increase in lesions in fetal liver and kidney. None of the increases in malformations, anomalies or pathological lesions reached statistical significance on a per litter basis, owing to the small number of litters per dose group (Wangikar et al., 2005).

2.2.5 Special studies

(a) Neurotoxicity

Ochratoxin A is neurotoxic in vitro and in vivo in rats given ochratoxin A orally for 7–35 days at doses of 120 and 290 µg/kg bw per day, as described previously by this Committee (Annex 1, reference *153*). More recent studies have been

published, most of which have been described by EFSA (European Food Safety Authority, 2006), and are briefly summarized below.

(i) In vitro

In cultured rat aggregated brain cells, 10–20 nmol ochratoxin A/l was shown to increase the expression of genes involved in the brain inflammatory system (messenger ribonucleic acid [mRNA] of peroxisome proliferator–activated receptor, haem oxygenase-1 and inducible nitric oxide synthase) and to reduce the expression of glial fibrillary acidic protein, a constituent of the intermediate filaments in astrocytes (Zurich et al., 2005).

In cultured rat embryonic midbrain cells, 0.5 and 1 µg ochratoxin A/ml caused a reduction in the number of viable cells, an induction of transcription factors activator protein-1 and nuclear factor-kappa B (NF-κB) activation as well as an inhibition of neurite outgrowth at the higher concentration (Hong et al., 2002).

In cultured proliferating neural stem/progenitor cells from adult mouse hippocampus, ochratoxin A added at concentrations of 0.01–100 µg/ml caused dose- and time-dependent (6–72 h) decreases in viability of both proliferating and differentiating cells. Proliferating cells were more vulnerable to ochratoxin A toxicity despite robust DNA repair and anti-oxidative responses. Preconditioning of the cells with the pro-oxidant diethyl maleate increased DNA repair activity and provided a moderate degree of neuroprotection. The authors speculated that these responses may contribute to impaired hippocampal neurogenesis in vivo (Sava et al., 2007).

(ii) In vivo

In mice, striatal dopamine was depleted in a dose-dependent manner following single intraperitoneal doses of 3–6 mg ochratoxin A/kg bw. Oxidative stress, oxidative DNA damage and a transient inhibition of oxidative DNA repair were also seen in the cerebellum, cortex, hippocampus, midbrain, caudate/putamen and pons/medulla (Sava et al., 2006).

Groups of 10 young (aged 12 weeks) and old (aged 27–20 months) female SPF Wag rats were given ochratoxin A by oral gavage at doses of 0, 70, 340 or 1680 µg/kg bw per day for 4 weeks. A significant increase in mortality occurred at the highest dose in both young and old rats. Vacuolation of the white brain matter (cerebellar medulla and ventral parts of the brain stem) was increased at all dose levels, reaching statistical significance in young rats at 340 and 1680 µg/kg bw per day and in old rats at 70 and 340 µg/kg bw per day (Dortant et al., 2001).

Groups of eight rats received 0 or 289 µg ochratoxin A/kg bw per day or the same dose of ochratoxin A plus melatonin (10 mg/kg bw per day) orally via the drinking-water for 4 weeks. Significant reductions in the concentrations of hippocampal *N*-methyl-D-aspartate receptor subunits 2A and 2B (involved in memory and learning processes) were reported in ochratoxin A–treated rats compared with controls. Melatonin partially protected against the reduction in *N*-methyl-D-aspartate receptor subunits caused by ochratoxin A (Delibas et al., 2003).

(b) Immunotoxicity

Ochratoxin A is immunotoxic in vitro and in vivo, as described previously by this Committee (Annex 1, reference *153*). More recent studies have been described by EFSA (European Food Safety Authority, 2006) and are briefly summarized below.

(i) In vitro

In rat lymphoid cells exposed to concentrations of ochratoxin A of 0, 0.5, 2 or 20 µmol/l (equivalent to 0, 0.2, 0.8 or 8 mg/l), the cytotoxic activity of natural killer cells was dose-dependently decreased, reaching statistical significance at the highest dose. The cytotoxic T lymphocyte activity was significantly decreased at the lowest concentration only. The bacteriolytic activity of macrophages varied only slightly, and the proliferative response of lymphocytes to concanavalin A and lipopolysaccharide was not affected (Alvarez-Erviti et al., 2005).

In a study using very high concentrations, ochratoxin A inhibited the mitogen (concanavalin A)–induced lymphocyte proliferation in purified lymphocytes from piglets. The IC_{50} was 1.3 µmol/l (equivalent to 0.52 mg/l), which is several orders of magnitude higher than the highest plasma levels found in humans (Keblys et al., 2004).

(ii) In vivo

Groups of 10 young (aged 12 weeks) and old (aged 27–20 months) female SPF Wag rats were given ochratoxin A by oral gavage at doses of 0, 70, 340 or 1680 µg/kg bw per day for 4 weeks. A significant increase in mortality occurred at the highest dose in both young and old rats. No old animals were available for testing of immune parameters at the highest dose because of mortality. Decreased immunoglobulin G (IgG) levels were seen at 340 µg/kg bw per day in both age groups and also at 1680 µg/kg bw per day in the young rats. Ochratoxin A also induced a dose-related reduction in the splenic T cell fraction of young rats, which was statistically significant at the highest dose only (Dortant et al., 2001).

Male Wistar rats were given ochratoxin A by oral gavage at doses of 0, 50, 150 or 450 µg/kg bw per day for 28 days, and several immune function assays were performed at the end of treatment. The study was conducted according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 407 (1995). The natural killer cell activity was strongly affected; the per cent lysis of the target cells was significantly reduced in all treated groups, and natural killer cell activity was completely suppressed at the highest dose. The response of splenocytes to sheep red blood cells was decreased in an apparently dose-dependent manner, but none of the reductions in response were statistically significant. The cytotoxic T lymphocyte activity was lowered in the 50 µg/kg bw per day group only. The bacteriocidal activity of macrophages was significantly reduced at 50 and 450 µg/kg bw per day, but not at the intermediate dose of 150 µg/kg bw per day. Histopathology of the thymus and spleen did not show significant differences from control (Alvarez et al., 2004).

(c) Mechanisms of toxicity in the kidney

Induction of collagen secretion (a marker of fibrosis) has been shown in the OK proximal tubular cell line and in cultured primary human renal proximal tubular cells exposed to ochratoxin A. Collagen secretion was both time and dose dependent, as was the induction of cell toxicity (Sauvant et al., 2005a).

Exposure of a rat kidney proximal tubular cell line (NRK-52E) to ochratoxin A at concentrations of 100 and 1000 nmol/l resulted in changes typical of chronic interstitial nephropathy, including loss of epithelial tightness, reduced cell number due to necrosis, as measured by release of lactate dehydrogenase (LDH) or DNA ladder formation, and apoptosis, as measured by capsase-3 activation. Ochratoxin A induced NF-κB activity (a marker of inflammatory activity), collagen secretion and generation of alpha smooth muscle actin (a marker of epithelial mesenchymal transition). It also induced three mitogen-activated protein kinases (MAPK): extracellular signal–regulated kinase 1 and 2 (ERK 1/2) (thought to be promitotic), c-jun *N*-terminal kinase (JNK) and extracellular-regulated protein kinase 38 (p38) (thought to be involved in apoptosis, fibrosis and inflammation). All three were induced in a dose-dependent manner (Sauvant et al., 2005b).

Exposure of OK and NRK-25E cells to ochratoxin A in the presence of an inhibitor of ERK 1/2 showed that the toxicity of ochratoxin A, as measured by cell number, protein, epithelial tightness, apoptosis and necrosis, was increased. Biomarkers of inflammation, fibrosis and epithelial mesenchymal transition were also enhanced in the presence of the inhibitor of ERK 1/2, compared with ochratoxin A alone. The authors speculated that the toxicity of ochratoxin A may be influenced by the balance between the profibrotic ERK 1/2 and the antifibrotic JNK and p38, which are not affected by inhibition of ERK 1/2, and that naturally occurring inhibitors of ERK 1/2, such as anthocyanidins, may amplify the effects of ochratoxin A (Sauvant et al., 2005c).

The effects of ochratoxin A on human kidney proximal tubule cells and on human lung fibroblasts in primary culture have been studied by the same group. The cells were exposed to ochratoxin A at concentrations of 0.3 nmol/l up to 10 µmol/l for 2, 5 or 14 days. The activities of capsase-3 and LDH were measured as indicators of apoptosis and necrotic cell death. Cellular protein content, collagen, fibronectin secretion and the activation of transcription factor NF-κB were also measured. Qualitatively similar effects were seen in the two types of cells tested, but kidney cells were around 10 times more sensitive than fibroblasts with respect to increases in capsase-3 and LDH release. Very low concentrations (0.3–10 nmol/l) of ochratoxin A led to hypertrophy in kidney cells exposed for 14 days. A fibrotic reaction was confirmed by increases in NF-κB activity, collagen III and fibronectin secretion in the kidney cells, which were absent in the fibroblasts. The authors commented that the changes seen in cultured kidney cells exposed for 14 days at low nanomole per litre or sub-nanomole per litre concentrations may indicate a need for reassessment of the effects of ochratoxin A consumption in humans, which generally leads to average blood concentrations around 1 nmol/l (Schwerdt et al., 2007).

(d) Epigenetic modes of action

(i) Oxidative DNA damage

Earlier work on oxidative stress, as a potential mechanism of ochratoxin A toxicity that could lead indirectly to damage to DNA and eventually tumorigenesis, has been summarized previously by this Committee (Annex 1, reference *153*). Commenting on the discrepant data on mode of action in 2001, the Committee noted that the oxidative stress hypothesis provided an alternative explanation to the hypothesis of direct damage to DNA and was more consistent with the observations. More recent data on oxidative stress are summarized below.

In vitro

Ochratoxin A–induced (oxidative) DNA damage, cytotoxicity (necrosis, growth inhibition, apoptosis) and modulation of GSH were investigated in cell lines (V79, CV-1) and in primary rat kidney (PRK) cells. After 24 h incubation, viability of V79 but not CV-1 cells was strongly decreased by ochratoxin A at concentrations above 2.5 µmol/l. Strong inhibition of growth was observed in both cell lines, with an IC_{50} around 2 µmol/l. Apoptosis was increased in CV-1 cells (V79 not tested) at concentrations above 1 µmol/l. Specific oxidative DNA damage (the difference between total DNA damage and basic DNA damage) was assessed by the comet assay after additional treatment with repair enzyme formamido-pyrimidine-DNA-glycosylase (Fpg) or endonuclease III, which recognize oxidized purine and pyrimidine bases, cut them out and nick the DNA in the respective sites, resulting in additional DNA damage. Specific oxidative DNA damage was induced in all cell systems by ochratoxin A at low concentrations, from 0.25 µmol/l upwards in V79 cells and from 1 µmol/l upwards in CV-1 and PRK cells. Basic DNA damage was not increased or was seen only at longer incubations or higher concentrations of ochratoxin A. Total GSH levels in CV-1 cells were significantly reduced after 1 h of incubation at 100 µmol/l, but slightly increased after 24 h. The authors concluded that the observations of oxidative DNA damage and cytotoxicity at low concentrations of ochratoxin A suggest that oxidative stress and a reactive proliferative response play a role in its renal carcinogenicity (Kamp et al., 2005a).

In a cultured human renal proximal tubular cell line, HK-2, a concentration-related increase in the level of ROS compared with controls was observed after 6 h exposure to ochratoxin A at 50–600 µmol/l. Pretreatment with *N*-acetyl-L-cysteine, an antioxidant ROS scavenger, decreased the level of ROS and increased cell survival. *N*-Acetyl-L-cysteine also reduced DNA damage at 50–200 µmol/l, as measured by the comet assay, but not at higher ochratoxin A concentrations of ≥400 µmol/l, at which the generation of ROS outpaced the reduction by *N*-acetyl-L-cysteine (Arbillaga et al., 2007a).

In a follow-up study, gene expression was assessed in HK-2 cells exposed to ochratoxin A at 50 µmol/l for 6 or 24 h. After 6 h, when slight cytotoxicity was evident (83% survival), genes involved in the mitochondrial electron transport chain were up-regulated. After 24 h, when there was greater cytotoxicity (51% survival), genes implicated in the oxidative stress response were also up-regulated. Increases

in the level of ROS and oxidative DNA damage as assessed by the comet assay were also evident at both time intervals and were more pronounced in the presence of higher cytotoxicity. There was no effect at either time interval on genes implicated in DNA damage response (repair, apoptosis and cell cycle control) (Arbillaga et al., 2007b).

Pig kidney epithelial (PK15) cells were exposed to ochratoxin A at concentrations of 0, 0.05, 0.5 and 5 µg/ml for 24 or 48 h. At the highest concentration, cell viability was reduced by 35% after 24 h and LPO increased by 56% and 85% at 24 and 48 h, respectively, compared with controls. GSH was significantly reduced by 18% after 24 h of exposure to 0.05 µg/ml (Klaric et al., 2007).

The effects of pretreatment with antioxidant catechins, epigallocatechin gallate and epicatechin gallate, on ochratoxin A toxicity were investigated in a pig kidney cell line (LLC-PK1) that has been used as a model for kidney toxicity. Pretreatment with catechins at 1–60 µmol/l was combined with subsequent exposure to 10–20 µmol ochratoxin A/l, and cell viability, ROS production and DNA fragmentation were measured. The pretreatment with catechins prevented ochratoxin A–induced cell death and reduced ochratoxin A–induced ROS production and DNA fragmentation. In tests of the antioxidant activity of the catechins alone, epigallocatechin gallate and epicatechin gallate showed good free radical scavenging power, in accord with their inhibition of ROS production (Costa et al., 2007).

In vivo

Ochratoxin A was shown to induce DNA strand breaks as assessed by comet assay in liver, kidney and spleen of Fischer 344 rats given doses of 0, 0.25, 0.50, 1.0 or 2.0 mg/kg bw per day for 2 weeks, 5 days/week. In liver and kidney, the extent of DNA damage was further enhanced in a dose-dependent manner in the presence of the repair enzyme Fpg, which converts oxidative DNA damage into strand breaks, suggesting the presence of oxidative DNA damage (Mally et al., 2005b).

The occurrence of oxidative DNA damage was investigated in vivo in male Fischer 344 rats dosed orally with ochratoxin A at 0, 0.03, 0.1 or 0.3 mg/kg bw per day for 4 weeks. The doses used were within the range known to have caused kidney tumours in rats in a 2-year study. Oxidative DNA damage was assessed by the comet assay, with and without use of the repair enzyme Fpg. There was no effect on basic DNA damage, but oxidative DNA damage was detected with Fpg treatment in kidney and liver at all doses tested (Kamp et al., 2005b).

Male Fischer 344 rats were fed ochratoxin A in the diet for up to 2 years at concentrations giving an initial intake at the initial body weight of 175 g of 300 µg/kg bw per day, declining and then held at 100 µg/kg bw per day when the animals reached a body weight of 333 g. Renal tumours appeared during the last 6 months of the study in 25% of the animals. The gene expression profile in the liver and kidney was studied in groups of five animals at intervals of 7 and 21 days and 4, 7 and 12 months after the commencement of treatment. In both organs, down-regulation of genes was the predominant effect. Two main clusters of genes could be identified that were continuously up-regulated or down-regulated over the five

time points studied. The kidney showed more modulation than the liver, possibly reflecting the elevated content and prolonged residence time of ochratoxin A in that organ. In the kidney, many genes expected to be induced by oxidative stress were significantly down-regulated, whereas no such modulation was seen in the liver; many of these genes share the antioxidant regulatory element in their promoter region, which is under transcriptional control by nuclear factor-erythroid 2 p45-related factor (Nrf2). Genes involved in fatty acid metabolism and cytochrome P450 were also selectively down-regulated in the kidney; many of these genes share the common promoter hepatocyte nuclear factor 4-alpha (HNF4α), and several genes from other functional classes also under transcriptional control by HNF4α were also down-regulated, as was the mRNA specific for HNF4α. Several genes known as markers of kidney injury and cell regeneration were up-regulated. Only small changes were seen in the expression of genes involved in DNA synthesis and repair or genes induced as a result of DNA damage. Very little change was seen in genes associated with apoptosis. There was a strong down-regulation of regucalcin, which is known to play a role in the regulation of intracellular calcium homeostasis in kidney tubule cells. The authors concluded that several epigenetic mechanisms may be involved in ochratoxin A carcinogenicity, including alteration of calcium homeostasis and disruption of pathways regulated by transcription factors HNF4α and Nrf2. In particular, depletion of Nrf2-regulated enzymes is likely to impair cellular defences against oxidative stress, but the authors acknowledge that there is no direct evidence of a link between the gene expression changes observed and kidney tumour formation (Marin-Kuan et al., 2006).

In a follow-up study on the same ochratoxin A–treated animals, it was confirmed that the loss of expression of Nrf2-regulated genes seen in the previous study (Marin-Kuan et al., 2006) was accompanied by decreased protein expression of several markers of the Nrf2-regulated gene battery in the kidney. Inhibition of Nrf2 activity was also shown in vitro by exposure of rat liver (primary hepatocytes and RL-34) cells and kidney (NRK) cells to ochratoxin A at concentrations of 1.5–6 µmol/l. Down-regulation of Nrf2 gene expression was accompanied by oxidative DNA damage both in vitro and in vivo, as measured by the formation of abasic sites in DNA. In vitro, the effects of ochratoxin A were prevented by pretreatment of the cell cultures with inducers of Nrf2 activity. The authors concluded that reduction of cellular defences against oxidative stress via Nrf2 inhibition may be a plausible mechanism for ochratoxin A nephrotoxicity and carcinogenicity (Cavin et al., 2007).

Studies on the protective effect of substances with antioxidant and free radical scavenging activities have provided further evidence of the possible role of oxidative stress in ochratoxin A–induced toxicity.

Pretreatment or co-treatment of rats with melatonin at doses of 10–20 mg/kg bw per day has a preventive effect on ochratoxin A–induced liver and kidney toxicity (Aydin et al., 2003) and on indicators of oxidative stress, such as changes in liver and kidney GSH peroxidase, superoxide dismutase and LPO, as measured by production of malondialdehyde after 4 weeks of ochratoxin A treatment (Meki & Hussein, 2001; Ozcelik et al., 2004; Abdel-Wahhab et al., 2005; Sutken et al., 2007).

Red wine has been shown to exert a protective effect against ochratoxin A nephrotoxicity in rats by limiting oxidative damage as measured by renal lipohydroperoxides, reduced and oxidized GSH and renal superoxide dismutase activity. The protective components in red wine may be the antioxidant flavonoids (Bertelli et al., 2005). The same group has shown that red wine reduces ochratoxin A–induced renal cortical fibrosis in rats during its early developmental stage by inhibiting the molecular mechanisms of the tubular cell transition from epithelial phenotype to myofibroblasts, which are the major effector cells responsible for deposition of interstitial extracellular matrix in pathological fibrosis. However, red wine has no effect on the mechanisms that predominate later to induce fibrosis under chronic ochratoxin A treatment (Gagliano et al., 2005). Conversely, others have shown, using an in vitro model, intestinal Caco-2/TC7 cells, that dealcoholated red wine at a 1:10 dilution in the medium may act synergistically with ochratoxin A (at a concentration of 100 µmol/l) to trigger an apoptotic cascade in such cells (Ranaldi et al., 2007).

The potential protective effect of 2-mercaptoethane sulfonate, which reduces oxidative stress by increasing free thiols in the kidney, on renal toxicity and carcinogenicity induced by ochratoxin A has been examined in a long-term rat study. 2-Mercaptoethane sulfonate significantly reduced karyomegaly but did not reduce the incidence of renal tumours (Pfohl-Leszkowicz & Manderville, 2007).

(ii) Effects on cell–cell signalling and cell division

In vitro

Since disruption of cell–cell signalling has been implicated in contributing to the mechanism of carcinogenicity of a number of nephrotoxic agents, the effects of ochratoxin A on gap junction intercellular communication (GJIC) and cell adhesion have been investigated. Such changes may, however, be the consequence rather than the cause of the toxicity of ochratoxin A. Madin Darby canine kidney (MDCK) cells were exposed for up to 24 h in culture to ochratoxin A at concentrations from 1 to 50 µmol/l. After 4 h exposure, when there was no evidence of cytotoxicity, GJIC was significantly affected, as measured by the reduction in distance of dye transfer, at 10 µmol/l and above. After 24 h exposure to 25 or 50 µmol ochratoxin A/l, there was almost complete inhibition of GJIC compared with 4 h, but this was accompanied by substantial cytotoxicity, suggesting disruption of monolayer integrity. However, inhibition of GJIC was also observed at lower, non-cytotoxic concentrations (1 and 10 µmol/l) after 24 h. Gap junctions, which allow exchange of signalling molecules between cells, are formed by oligomerization of connexin (Cx) proteins. Inhibition of GJIC by ochratoxin A was associated with a small, but dose-dependent, decrease in Cx43 protein levels at all time points, probably via down-regulation of Cx43 expression, rather than by changes in total cellular phosphorylation. Intracellular amounts of the adherens junction proteins, E-cadherin and β-catenin, were not affected by ochratoxin A. Changes were seen in localization of these two adherens at 25 and 50 µmol/l, in association with cytotoxicity, but it was not possible to determine whether these changes may have

preceded cell death. The authors concluded that disruption of cell–cell signalling may contribute to the toxicity and carcinogenicity of ochratoxin A (Mally et al., 2006).

Immortalized human kidney epithelial cells, a model in which the morphological changes closely resemble those in the rat kidney in vivo, were exposed to ochratoxin A at concentrations of 0, 1, 10 and 50 µmol/l. Significant time- and dose-dependent changes were observed at concentrations of 10 and 50 µmol/l. These concentrations are in the same range as plasma concentrations after repeated administration of ochratoxin A to rats at doses known to induce tumours. The changes included increases in apoptosis and decreases in the rate of mitosis. Nuclear morphology showed that the later stages of mitosis (anaphase/telophase) were rarely present, and the number of aberrant nuclei with misaligned chromosomes was significantly increased at 10 and 50 µmol/l. There was also formation of giant cells with abnormally large or multiple nuclei, sometimes still connected by chromatin bridges, which is indicative of defects in chromosome segregation and/or cytokinesis. Irregular, asymmetric and sometimes multipolar mitotic spindles were also seen. Disruption of cell–cell and cell–matrix contacts was also observed, associated with decreased phosphorylation of focal adhesion kinase, a central component of integrin-mediated cell adhesion and survival signalling. At the same time, increased activation of the stress-activated MAPK, JNK and the antiapoptotic transcription factor NF-κB was observed. At 50 µmol/l, increased phosphorylation of ERK 1/2 was also observed. To further investigate whether the abnormal mitotic spindles were due to ochratoxin A interference with microtubule polymerization, an in vitro tubulin polymerization assay was conducted in a cell-free environment. Ochratoxin A was found to inhibit microtubule assembly at 200 and 400 µmol/l but not at 50 or 100 µmol/l. The authors hypothesized that the mechanism by which ochratoxin A promotes tumour formation involves interference with microtubule dynamics and mitotic spindle formation, resulting in apoptosis or premature exit from mitosis. This causes blocked or asymmetric cell division, which may favour the occurrence of cytogenetic abnormalities, ultimately leading to tumour formation (Rached et al., 2006).

In vivo

Kidney samples were taken from male Fischer 344 rats fed ochratoxin A in the diet at a dose of 300 µg/kg bw per day for 7 days, 21 days or 12 months and analysed for various cell signalling proteins known to be potentially involved in kidney carcinogenicity. This dose was known to have caused kidney tumours if fed over 2 years (Mantle et al., 2005). Ochratoxin A was found to increase the phosphorylation of atypical protein kinase C (PKC) at all time points compared with controls, the increases being statistically significant at 21 days and 12 months. This was correlated with a selective downstream activation of the MAPK extracellular signal–regulated kinases isoforms 1 and 2 (ERK 1/2) and of their nuclear substrate ETS-domain protein 1 (ELK 1/2) and cytosolic substrate, ribosomal-S6 kinase (p90RSK). Analysis of effectors acting upstream of PKC indicated a possible mobilization of the insulin-like growth factor-1 receptor (IGF-1r) and phosphoinositide-dependent kinase-1 system (PDK1). Increased histone deacetylase (HDAC) enzymatic activity associated with enhanced HDAC3 protein

expression was also observed. The authors noted several associations between the changes observed in this rat study and events that have been linked with human renal cancer in other studies: i.e. IGF-1 and subsequent MAPK downstream responses, activation of the MAPK-ERK cascade, increases in IGF-1r and increased atypical PKC activity leading to MAPK activation, which is thought to be a key element in the development of kidney cell carcinoma consequent to inactivation of the tumour suppressor gene von Hippel-Lindau gene product. IGF-1r is also thought to be associated with tumour development in rodents, whereas activation of PKC activity and selective mobilization of ERK 1/2 have been linked to renal cancer in rats. The authors concluded that these results suggest that chronic dietary administration of ochratoxin A to male rats may result in an MAPK response compatible with a selective stimulation of proliferation and stress response, rather than induction of apoptosis, as suggested by earlier in vitro studies (Marin-Kuan et al., 2007).

Wild-type rats and Eker rats heterozygous for a dominant germline mutation in the tuberous sclerosis 2 (*Tsc2*) tumour suppressor gene were exposed orally to ochratoxin A by gavage at a dose of 210 µg/kg bw per day for 1, 3, 7 or 14 days. Renal cell histopathology, cell proliferation and gene expression profiles were investigated in the renal cortex/outer medulla. Ochratoxin A caused slight cortical pathology (preneoplastic lesions) and significantly increased cell proliferation in both strains of rat. A number of genes in the phosphatidylinositol 3-kinase-AKT-Tsc2-mammalian target of rapamycin signalling were down-regulated. Eker rats were more sensitive than wild-type rats for all effects. The overall pattern of effects suggested that *Tsc2* may be involved in ochratoxin A toxicity (Stemmer et al., 2007).

2.3 Observations in humans

2.3.1 Biomarkers of exposure

(a) Blood

As the Committee has previously commented, ochratoxin A has a half-life of about 35 days in humans, and the blood concentrations are considered to represent a convenient biomarker of exposure during previous weeks. This biomarker has been used extensively in epidemiological studies. Similar estimates of exposure have been derived from dietary surveys and from blood analyses, suggesting that the latter is a reliable biomarker (Annex 1, reference *153*).

In the Committee's previous evaluation (Annex 1, reference *153*), the concentrations of ochratoxin A in blood samples from healthy persons (n = 3717), obtained in surveys conducted in 17, mainly European, countries, ranged between 0.1 and 40 ng/ml (with an exceptional maximum of 160 ng/ml). The results of more recent surveys, summarized in Table 5, suggest a possible decline in extreme peak values for blood concentrations compared with earlier surveys.

Table 5. Recent studies on the occurrence of ochratoxin A in blood samples of healthy humans

Country	Collecting period	Number of positive samples (%)	Detection limit (ng/ml)	Mean plasma concentration (ng/ml)	Reference
Italy	1995–1996	134/138 (97)	0.1	0.56	Palli et al. (1999)
Croatia	1997–1998	468/983 (48)	0.2	0.30	Peraica et al. (2001)
Norway, Sweden	1997–1998	393/393 (100)	0.01	0.18–0.21	Thuvander et al. (2001)
Morocco	2000	185/309 (60)	0.1	0.29	Filali et al. (2002)
Lebanon	2001–2002	82/250 (33)	0.1	0.17	Assaf et al. (2004)
Poland	1998–1999	30/30 (100)	0.02	1.14	Postupolski et al. (2006)
Chile	2004	62/88 (70)	0.1	0.42–0.88	Munoz et al. (2006)
Argentina (two cities)	2004 2005	125/199 (63) 151/236 (64)	0.012 0.43	0.15	Pacin et al. (2007)

A recent review has considered how exposure to ochratoxin A might relate to the occurrence of Balkan endemic nephropathy, by comparing blood levels in rural endemic versus non-endemic regions of Balkan countries, while acknowledging that systematic data are not available. The authors note that although some studies have indicated that contamination of food by ochratoxin A may be more widespread in rural endemic areas than in non-endemic areas, this is not reflected by markedly elevated blood concentrations of ochratoxin A. Moreover, blood concentrations of ochratoxin A in the same range as those observed in endemic areas have been found in countries with no history of endemic nephropathy. These authors also noted that human blood concentrations of ochratoxin A, even in areas with relatively high dietary exposure, are (at least 2) orders of magnitude below the mean concentration of ochratoxin A in the blood of rats that is known to cause nephrotoxicity and kidney tumours with long-term treatment (Mally et al., 2007).

(b) Urine

Recent advances in analytical methods have enabled monitoring of the generally low levels of ochratoxin A in urine.

A total diet study has been conducted in the United Kingdom on 50 individuals, in which composite duplicate-diet samples and plasma and urine samples were analysed over a period of 30 days. The affinity column methodology used achieved sensitivities of 0.001 ng/g in composite foods, 0.1 ng/ml in plasma and 0.01 ng/ml in urine. A significant correlation was found between ochratoxin A intakes and concentrations in urine. Urine was found to be a better indicator of ochratoxin A intakes than plasma, confirming urine as a suitable biomarker to

estimate exposure to ochratoxin A. Urine concentrations in 46/50 samples ranged from <0.01 to 0.058 ng/ml (Gilbert et al., 2001).

In Italy, ochratoxin A was detected in 22 out of 38 urine samples from healthy people in a range of 0.012–0.046 ng/ml (Pascale & Visconti, 2001).

The ochratoxin A content of urine samples from 88 healthy humans living at five settlements in three counties in Hungary was determined. Ochratoxin A was detected in 61% of the samples at an average concentration of 0.013 ng/ml (range: 0.006–0.065 ng/ml) (Fazekas et al., 2005).

Ochratoxin A was detected in 42 out of 60 urine samples taken from healthy persons in Coimbra, Portugal. The concentrations ranged from 0.021 to 0.105 ng/ml, with an average of 0.038 ng/ml (limit of quantification 0.02 ng/ml) (Pena et al., 2006).

(c) Milk

Ochratoxin A has been found in human milk at a wide range of concentrations (see Table 6).

Table 6. Studies on the occurrence of ochratoxin A in human milk samples

Country	Number of positive samples (%)	Range of concentration in milk[a] (ng/ml)	Reference
Germany	4/36 (11%)	0.017–0.030	Gareis et al. (1988)
Italy	9/50 (18%)	1.2–6.6	Micco et al. (1991)
Sweden	23/40 (57%)	0.01–0.04	Breitholtz-Emanuelsson et al. (1993)
Sweden	39/39 (100%)	0.09 (average)	Breitholtz-Emanuelsson et al. (1993)
Hungary	38/92 (41%)	0.02–7.3	Kovacs et al. (1995)
Sierra Leone	35/40 (87%)	0.2–337	Jonsyn et al. (1995)
Australia	2/100 (2%)	3–3.6	Apostolou et al. (1998)
Norway	17/80 (21%)	10–182	Skaug et al. (2001)
Italy	198/231 (87%)	6.01 (average)	Turconi et al. (2004)
Brazil	2/50 (4%)	0.01 and 0.2	Navas et al. (2005)
Poland	5/13 (38%)	0.005–017	Postupolski et al. (2006)
Egypt	36/50 (72%)	1.89 (average)	Hassan et al. (2006)

[a] Unless stated as an average.

Recent evidence suggests that excretion of ochratoxin A into human milk is mediated by breast cancer resistance protein (BCRP). BCRP is a member of the ATP-dependent efflux transporter family, which is highly expressed during lactation in various species, including humans, and is known to be responsible for the excretion of various drugs and xenobiotics into milk (Jonker et al., 2005; Schrickx

et al., 2005; Van Herwaarden et al., 2006). This excretion serves to reduce maternal body burdens but means that breastfed infants are exposed to ochratoxin A.

2.3.2 Biomarkers of effect

Raised urinary β2-microglobulin levels have been reported in association with impairment of renal tubular function, correlating with high levels of ochratoxin A exposure in patients with chronic interstitial nephropathy living in an area of Tunisia known to have high dietary exposure to ochratoxin A (Hassen et al., 2004).

2.3.3 Epidemiological studies

The earlier literature on the association between human exposure to ochratoxin A via the diet and the occurrence of Balkan endemic nephropathy and associated urinary tract tumours was summarized by the Committee in its previous evaluation (Annex 1, reference *153*). However, contrary to the clear causal evidence of ochratoxin A–induced nephrotoxicity and nephrocarcinogenicity in rodents (Annex 1, reference *153*; O'Brien & Dietrich, 2005; European Food Safety Authority, 2006), the significance of ochratoxin A for human health still remains unclear from the available epidemiological evidence (Fink-Gremmels, 2005; European Food Safety Authority, 2006). Moreover, ochratoxin A exposure is only one of several hypotheses concerning an environmental etiology for Balkan endemic nephropathy; exposure to aristolochic acid from the seeds of a weed (*Aristolochia clematis*) that contaminates wheat in endemic regions and intake of potentially carcinogenic organic compounds via drinking-water from the occurrence of lignites from Pliocene-age coals in endemic regions have also been considered as potential causes of Balkan endemic nephropathy and urinary tract tumours (Stefanovic et al., 2006).

Kidney function in infants breastfed for at least 4 months has been assessed in relation to maternal and infant serum and maternal milk concentrations of ochratoxin A. Thirty-six (72%) of the 50 mothers included in the study had detectable ochratoxin A in serum and milk. Infants with higher ochratoxin A levels in their serum ($\geq$2 ng/ml) had higher levels of urinary β2-microglobulin and microalbuminuria than did infants with lower serum levels (<2 ng/ml). Both differences were statistically significant with the use of univariate analysis. Multivariate logistic regression analysis showed that there was a significant correlation between a higher infant serum level of ochratoxin A and the degree of microalbuminuria, but not β2-microglobulinuria (Hassan et al., 2006).

The possible role of a genetic predisposition to toxicity from ochratoxin A has been investigated in Jelma, Tunisia, an area in which it is known that there is high dietary exposure to ochratoxin A and in which chronic interstitial nephropathy of unknown etiology with similarities to Balkan endemic nephropathy is often seen. Twenty-one people in four rural families, all exposed to ochratoxin A via the diet, were tested for urinary β2-microglobulin concentration, ochratoxin A concentration in blood, urine and food and human histocompatibility leukocyte antigen (HLA) haplotype. Ochratoxin A was detectable in blood and urine in 19 out of the 21 persons at concentrations in blood ranging from 8 to 1468 ng/ml. Three siblings in

one family had chronic interstitial nephropathy (altered renal function, β2-microglobulinuria and renal karyomegaly confirmed by histology). Haplotyping showed that the three with chronic interstitial nephropathy shared elements of the phenotype A3, B27/35 and DR7 (Creppy et al., 2005).

Familial clustering of Balkan endemic nephropathy has prompted a number of other genetic investigations. Limited studies on genetic polymorphisms in xenobiotic metabolizing enzymes in Bulgarian Balkan endemic nephropathy patients and their healthy relatives have shown a significantly higher risk for Balkan endemic nephropathy in individuals carrying the CYP3A5*1 allele, but the possible role of CYP3A5 activity in the development of Balkan endemic nephropathy is not known. A higher frequency of *GSTM1* wild-type allele (conferring a high activity of GSH transferases) has also been found in Balkan endemic nephropathy patients compared with controls, while the Multidrug Resistance 1 (*MDR1*) haplotype is associated with a lower frequency of Balkan endemic nephropathy (Stefanovic et al., 2006).

3. ANALYTICAL METHODS

Ochratoxin A is a colourless crystalline compound with blue fluorescence under ultraviolet light. It has a weak acidic character, with pK_a values of 4.4 (carboxyl group of phenylalanine) and approximately 7.5 (phenolic group). Acid hydrolysis splits ochratoxin A into phenylalanine and ochratoxin *alpha*, an optically active lactone acid. The lactone ring may be opened by alkali or treatment with some microbial esterases, and this removes toxicity. This reaction is reversible, under acidic conditions (Monaci & Palmisano, 2004).

Methods for analysing ochratoxin A were thoroughly reviewed in the previous report (Annex 1, reference *153*). At that time, it was reported that validated analytical methods were available for the determination of ochratoxin A in maize, barley, rye, wheat, wheat bran, wholemeal wheat, roasted coffee, wine and beer. The best methods used LC following cleanup using immunoaffinity columns. Over 40 methods were reviewed. This review is limited to recent developments.

3.1 Sample preparation

Sample preparation may involve either a milling or a wet slurry technique. A recent study has evaluated these two processes using 10-kg samples of cocoa, green coffee, almonds and pistachios and has shown that the wet slurry process has a lower coefficient of variation than dry milling (Spanjer et al., 2006). The investigators concluded that this was due to the smaller particle size produced by the wet slurry process and that this method of sample preparation was to be preferred.

3.2 Extraction

Traditionally, extraction has been by conventional liquid–liquid methods (Annex 1, reference *153*). Recently, a number of methods for solid-state extractions have been published. These typically use derivatized silica with C_8, C_{18} and

cyanopropyl (CN) stationary phases (Monaci & Palmisano, 2004). In a typical solid-phase extraction, column conditioning is followed by application of the liquid sample. Most interferences can be removed by a washing step. The analyte is then eluted with an organic solvent and, after drying, dissolved in a small amount of the mobile phase (Monaci & Palmisano, 2004). This procedure is relatively non-selective, and the cleanup levels achieved may be insufficient for some of the more challenging matrices that commonly contain ochratoxin A, including coffee, beer and wine. Antibody-based immunoaffinity columns are commonly used after solid-state cleanup for samples of these commodities.

For many solid samples, a solvent extraction step is still necessary. Extraction with acidic chloroform, acidic ethyl acetate containing 2 mol sodium chloride/l or methanol–sodium carbonate (1%), followed by back-extraction to sodium bicarbonate, has been recommended (Monaci & Palmisano, 2004). After washing the column, ochratoxin A can be eluted with methanol and, if necessary, further purified through an immunoaffinity column.

Immunoaffinity columns are expensive, and recently the use of more stable imprinted polymers has been described. With careful design, imprinted polymers have been reported to be very promising and cheaper alternatives to both solid-phase extraction and immunoaffinity columns (Monaci & Palmisano, 2004).

3.3 Ochratoxin A estimation

High-performance liquid chromatography (HPLC) remains the most popular method for determination of ochratoxin A. Most HPLC methods use a reversed-phase column and an acidic mobile phase, so the carboxyl group of the mycotoxin is in the undissociated form. The post-column addition of 10% ammonia solution increases the fluorescence emission of ochratoxin A by a factor of 10 (Monaci & Palmisano, 2004).

Recent advances have included the coupling of HPLC to a mass spectrometer using electrospray ionization (Timperio et al., 2006; Ventura et al., 2006).

TLC is still regarded as a cheap and effective technique for ochratoxin A estimation, particularly because of its low cost and adaptability. New methods include extraction with a mixture of phosphoric acid and dichloromethane and purification by liquid–liquid partitioning into sodium hydrogen carbonate, before separation by normal-phase TLC and detection by fluorescence as usual (Pittet & Royer, 2002); and extraction with a mixture of methanol and aqueous sodium bicarbonate solution, followed by partitioning into toluene before TLC (Ventura et al., 2005).

A number of recent papers deal with immunoassay techniques, especially various enzyme-linked immunosorbent assays (ELISA). Detection limits for HPLC and ELISA are quite comparable, but the ELISA technique suffers from false-positive results, because of cross-reactions, and, more importantly, false-negative results, especially from tissue extracts (Monaci & Palmisano, 2004).

Validation of mycotoxin methods has become very important, because they are used for regulatory purposes throughout the world. A list of known validated or official methods for ochratoxin A analysis is given in Table 7.

Table 7. Validated and official analytical methods for determining ochratoxin A in food samples[a]

Food	Cleanup	Recovery (%)	RSD (%) within lab	RSD (%) between labs	Reference
Baby food	IAC	108	18–36	29–63	Burdaspal et al. (2001)
White wine	IAC	88–105	7–11	13–16	Visconti et al. (2001)
Red wine	IAC	84–93	7–11	14–14	Visconti et al. (2001)
Beer	IAC	87–95	5–17	15–26	Visconti et al. (2001)
Roasted coffee	SPE + IAC	65–97	2–22	14–26	Entwisle et al. (2001)
Barley	IAC	65–113	4–24	12–33	Entwisle et al. (2000)
Barley	CC	64–72	21	23–28	Larsson & Moller (1996)
Wheat	IAC	91	10–13	14–32	Scott (2002)
Wheat bran	CC	64–72	17	20–28	Larsson & Moller (1996)
Rye	CC	64–72	12	18–31	Larsson & Moller (1996)
Maize	CC	53–97	20	21–32	Nesheim et al. (1992)
Pig kidney tissue	CC	53–97	16	33–68	Nesheim et al. (1992)
Cocoa	IAC	–	–	15–40	Brera et al. (2005)

CC, column chromatography; IAC, immunoaffinity column; RSD, relative standard deviation; SPE, solid-phase extraction.

[a] Modified from Monaci & Palmisano (2004).

4. SAMPLING PROTOCOLS

Sampling for analysis is always very important, but often neglected. Recently, Vargas et al. (2006a, 2006b, 2006c) published extensive information on the parameters governing sample size for testing green coffee for ochratoxin A.

5. LEVELS AND PATTERNS OF CONTAMINATION OF FOOD COMMODITIES

At the fifth-sixth meeting of JECFA (Annex 1, reference *152*), it was reported that ochratoxin A is produced by a single *Penicillium* species, *P. verrucosum*, by *Aspergillus ochraceus* and several related *Aspergillus* species, and by *A. carbonarius*, with a small percentage of isolates of the closely related species *A.*

niger. Since that report, some other *Aspergillus* and *Penicillium* species have been described as potential sources of ochratoxin A. One addition is that of *Penicillium nordicum*, recognized as a second *Penicillium* species able to produce ochratoxin A (Larsen et al., 2001). The latest information also indicates that *A. ochraceus* is an uncommon fungus, and isolates do not often produce ochratoxin A. However, two recently described species, *A. westerdijkiae* and *A. steynii*, split off from *A. ochraceus*, are the major producers. The following sections deal with these species in some detail.

5.1 Aspergillus *species that produce ochratoxin A*

Aspergillus species producing ochratoxin A can be divided into two distinct groups. The first includes species producing conidia coloured golden brown, classified in *Aspergillus* section *Circumdati* and related to *A. ochraceus*. The second includes species producing black conidia, classified in *Aspergillus* section *Nigri*, of which *A. carbonarius* is the most important.

5.1.1 Species in Aspergillus *section* Circumdati

Ten species classified in section *Circumdati* were reliably reported to produce ochratoxin A by Frisvad et al. (2004b). Most are rare in nature or at least uncommon in foods and not likely to contribute to the level of ochratoxin A in the diet. *Aspergillus cretensis*, *A. flocculosus*, *A. pseudoelegans*, *A. roseoglobosus*, *A. sulphureus* and *A. muricatus* (sexual state *Neopetromyces muricatus*) all fit into that category. *Aspergillus alliaceus* (sexual state *Petromyces alliaceus*), not usually classified in section *Circumdati*, but morphologically belonging there, also produces ochratoxin A. It is not found in foods.

Aspergillus sclerotiorum is a somewhat more common species in foods, but isolates produce only low levels of ochratoxin A (Frisvad et al., 2004b). *Aspergillus ochraceus*, traditionally regarded as a common species with rather variable morphology, has recently been divided into three species. *Aspergillus ochraceus* sensu stricto is now regarded by Frisvad et al. (2004b) as rather uncommon. Its role in that restricted sense may or may not be important in human health terms. Two recent segregates from *A. ochraceus* are now considered to be the major species in *Aspergillus* section *Circumdati* contributing ochratoxin A to the human diet.

The first of these species is *A. westerdijkiae*; according to Frisvad et al. (2004b), this is the correct name for the original "*A. ochraceus*" from South Africa that led to the discovery of ochratoxins. *Aspergillus westerdijkiae* differs from *A. ochraceus* by its inability to grow at 37 °C and by producing white to cream sclerotia rather than the purple ones of *A. ochraceus* (Frisvad et al., 2004b). *Aspergillus westerdijkiae* is a common species and a high producer of ochratoxin A (Frisvad et al., 2004b). The second new species, *A. steynii*, also differs from *A. ochraceus* by its inability to grow at 37 °C and from *P. westerdijkiae* by producing ellipsoidal, not spherical, conidia—and from both species by producing pale yellow, not golden brown or ochre, conidia (Frisvad et al., 2004b). Most *P. steynii* isolates produce large amounts of ochratoxin A, and it is a quite commonly occurring species (Frisvad et al., 2004b).

Much biochemical work reportedly carried out on *A. ochraceus* has really been on *A. westerdijkiae* (Frisvad et al., 2004b), but these three species are sufficiently similar that that work retains its value. The name *A. ochraceus* is likely to continue to be used for all three of these species in many reports in the future.

5.1.2 Species in Aspergillus *section* Nigri

Samson et al. (2004) reported that four species in *Aspergillus* section *Nigri* (the black Aspergilli) are capable of producing ochratoxin A. Two of these species were described as new by those authors. One of these, *A. sclerotioniger*, is known only from a single isolate. The second, *A. lacticocoffeatus*, is also very uncommon and may represent only a pale colour mutant of *A. niger*. All three known isolates of *A. lacticocoffeatus* produce ochratoxin A, however. The major producer of ochratoxin A among the black Aspergilli is now well established to be *A. carbonarius*. This is an old, well known and well defined species, but reports of its ability to produce ochratoxin A first appeared only in 1995 (Horie, 1995). This ability has been substantiated by many authors since (see Samson et al., 2004 for review). Virtually all known isolates of this species produce ochratoxin A.

The fourth ochratoxin A–producing species is *A. niger*, but only a very low percentage (6% or less) of true *A. niger* strains make ochratoxin A (Abarca et al., 2003; Pardo et al., 2004; and many more recent studies; see Samson et al., 2004 for review).

Other black Aspergilli of common occurrence in foods are *A. japonicus*, distinguished by microscopic appearance (only phialides are produced), and *A. tubingensis*, distinguished from *A. niger* by molecular methods. Neither of these species makes ochratoxin A (Samson et al., 2004).

5.2 Penicillium *species that produce ochratoxin A*

Since the previous report (Annex 1, reference *153*), some isolates previously considered to be *Penicillium verrucosum* have been segregated on various taxonomic grounds into a separate species, *P. nordicum*. Larsen et al. (2001) reported that *P. nordicum* isolates also produce ochratoxin A. *Penicillium verrucosum* and *P. nordicum* are classified in *Penicillium* subgenus *Penicillium* and are very closely related in appearance and physiology. However, while *P. verrucosum* is of common occurrence in cereals from cold climates, *P. nordicum* has been reported to occur in dried proteinaceus foods such as salami, ham and cheese (Frisvad et al., 2004a). On current evidence, *P. nordicum* appears to be a minor source of ochratoxin A in foodstuffs by comparison with *P. verrucosum*.

Although many other *Penicillium* species have been reported to make ochratoxin A, none of these reports is considered to be correct (Frisvad et al., 2004a).

5.3 Physiology and ecology of fungi that produce ochratoxin A

The three major groups of fungi producing ochratoxin A have quite different physiology and consequently quite different ecological habitats. To understand the

kinds of foods in which ochratoxin A occurs or to predict the potential for ochratoxin A formation, it is necessary to understand the physiology and ecology of these species and the differences between them. This information is outlined below.

5.3.1 Penicillium verrucosum *and* P. nordicum

Penicillium verrucosum is a slow-growing species under any conditions, but capable of growth at low water activity (a_w) (down to 0.80) and at low temperature (range 0–31 °C, optimum 20 °C) (Pitt & Hocking, 1997). The physiology of *P. nordicum* has been little studied, but, as it is closely related to *P. verrucosum*, it can be expected to have similar physiological characteristics. In particular, it grows poorly, if at all, at 30 °C, like *P. verrucosum* (Samson & Frisvad, 2004).

A notable feature of the ecology of *P. verrucosum* is that it grows only at lower temperatures. This results in a distribution that is apparently confined to cool temperate regions. Cereal crops are its major food habitat, and it ranges across northern and central Europe and Canada. It appears to be uncommon, indeed almost unknown, in warm climates or in other kinds of foods. The occurrence of this species in European cereals has two consequences: ochratoxin A is present in many kinds of European cereal products, especially bread and flour-based foods, and it is present in animals that eat cereals as a major dietary component. Ochratoxin A was detected in Danish pig meats 25 years ago (Krogh et al., 1973), and its implications for human and animal health were recognized at the same time. As bread and other cereal products and pig meats are major components of the European diet, the further consequence is that most Europeans who have been tested have shown appreciable concentrations of ochratoxin A in their blood (Hald, 1991; and many more recent reports). There is no doubt that this results from the growth of *P. verrucosum* in cereals.

In studying the mycoflora of grapes used for the production of a particular kind of dessert wine, Torelli et al. (2006) reported on the identification of 379 strains of *Penicillium* species isolated from the grapes. None was *P. verrucosum* or *P. nordicum*; this report reinforces the belief that *Penicillium* species are not responsible for the presence of ochratoxin A in grapes or grape products.

Penicillium nordicum has been reported to occur in meat products (salami, ham, sausage, chicken meat) and in cheese (Samson & Frisvad, 2004). It appears to be much more widely distributed than *P. verrucosum*, but nearly all reported sources have been products kept under refrigeration during manufacture. There have been few studies on its ecology. However, during a 1-year survey of seven ham manufacturing plants in Europe, Battilani et al. (2007) isolated potential mycotoxin-producing fungi from factory air and from the surface of the curing hams. More than 90% of the 19 000 fungal isolates examined were *Penicillium* species. When 300 of these isolates, randomly sampled, were identified to species, 10% were *P. nordicum*. When these 30 isolates were tested for ochratoxin A production on laboratory media, 50% were positive. Escher et al. (1973) had reported ochratoxin production on dry cured hams; an explanation is suggested by Battilani et al. (2007).

5.3.2 Aspergillus ochraceus *and close relatives*

Aspergillus ochraceus can be described as a mesophilic xerophile. Growth occurs between 8 °C and approximately 40 °C, with the optimum at 24–31 °C (Pitt & Hocking, 1997). Optimal conditions for growth are 0.95–0.99 a_w, whereas the lower limit for growth is 0.79 a_w on media containing sugars and down to 0.81 a_w on media based on sodium chloride. *Aspergillus ochraceus* grows slowly at pH 2.2 and well between pH 3 and 10 (Pitt & Hocking, 1997). Little is known about the physiology of *A. westerdijkiae* or *P. steynii*, except that they do not grow at 37 °C (Frisvad et al., 2004b). Other parameters governing growth can be expected to be similar to those for *A. ochraceus*. Indeed, some published data on *A. ochraceus* physiology have certainly used strains now classified as one of these two species (Frisvad et al., 2004b).

Because *A. westerdijkiae* and *A. steynii* have been distinguished from *A. ochraceus* only recently, the reported ecology of *A. ochraceus* is actually that of these three species. They have been isolated from a wide range of food products, but are more common in dried and stored foods than elsewhere. Stored foods from which these species (reported as *A. ochraceus*) have been isolated include smoked or salted dried fish, dried beans, biltong, soybeans, chickpeas, rapeseed, pepper, dried fruit and sesame seeds. Nuts are also a major source, especially pecans and pistachios, as well as peanuts, hazelnuts and walnuts. Reports from cereals and cereal products have been infrequent, but records include rice, barley, maize, wheat, flour and bran. These species have also been reported from cheese, spices, black olives, cassava and processed meats. However, none of these species is a common cause of food spoilage, so their presence is not a good indicator of significant mycotoxin production (Pitt & Hocking, 1997).

Numerous studies have detected *A. ochraceus* (and related species) in green coffee beans (e.g. Levi et al., 1974; Cantafora et al., 1983; Studer-Rohr et al., 1994; Taniwaki et al., 2003). Although these species are one source of ochratoxin A in coffee (Taniwaki et al., 1999), studies indicate that *A. carbonarius* is a more important source in coffee from most producing countries.

Aspergillus ochraceus has been isolated from a variety of Southeast Asian commodities, including maize, peanuts, soybeans and other beans, cashews and sorghum. Its presence or absence in any sample probably was related to length of storage rather than to geographical location or other factors (Pitt et al., 1993, 1994, 1998).

5.3.3 Aspergillus carbonarius *and* A. niger

The ability of *A. carbonarius* to produce ochratoxin A was first reported only 12 years ago (Horie, 1995; Téren et al., 1996; Varga et al., 1996; Heenan et al., 1998), but its importance is now well recognized. This species resembles *A. niger* in many features, and indeed the two species are very closely related. *Aspergillus carbonarius* differs from *A. niger* most notably in the production of larger spores, although other minor morphological differences exist. *Aspergillus carbonarius* grows at rather lower temperatures than *A. niger*, with a maximum around 40 °C and optimal conditions at 30 °C (Leong et al., 2006a; Romero et al., 2007). The

ability to grow at reduced a_w is also more restricted: germination occurs down to 0.85 a_w at 25 and 30 °C (Romero et al., 2007). Maximum ochratoxin A production has been reported to occur at 15 °C and 0.95–0.98 a_w (Estaban et al., 2006a; Leong et al., 2006a). Little ochratoxin A was produced above 25 °C (Leong et al., 2006a). Ochratoxin A was produced over a very wide pH range of 2 or 3–10 (Estaban et al., 2006b).

Aspergillus niger grows optimally at relatively high temperatures, with a growth minimum of 6–8 °C, a maximum of 45–47 °C and optimal conditions at 35–37 °C. *Aspergillus niger* is a xerophile, with germination reported at 0.77 a_w at 35 °C. Growth rates vary only slightly on media based on sugars, sodium chloride or glycerol or on media of pH 4.0 and 6.5, at various water activities; in other words, growth of *A. niger* appears to be little affected by food type. *Aspergillus niger* is able to grow down to pH 2.0 at high a_w (Pitt & Hocking, 1997).

Several other black *Aspergillus* species commonly occur in foods, especially *A. tubingensis* and *A. japonicus*, but these species do not produce ochratoxin A. Their physiology appears to be broadly similar to that of *A. niger.*

Aspergillus carbonarius, *A. niger* and *A. japonicus* occur together in foods and superficially look similar, so many surveys of Aspergilli in foods have not differentiated these three species, calling all black Aspergilli *A. niger*.

The black *Aspergillus* species have a high resistance to sunlight and ultraviolet light (Rotem & Aust, 1991; Leong et al., 2006b) and grow at relatively high temperatures. They are also very acid tolerant and prefer a somewhat reduced water activity (Leong et al., 2006a). For all these reasons, a major habitat for the black Aspergilli is in grapes (Snowdon, 1990), but it has not been shown that *Aspergillus* species are primary pathogens. The evidence is that they are able to invade only damaged grapes (Snowdon, 1990). Damage can be caused by the growth of pathogenic fungi such as *Botrytis cinerea*, *Rhizopus stolonifer* or powdery mildew or from skin splitting due to rain damage before harvest. Inclusion of damaged grapes at harvest or delays in crushing, conditions that permit continued growth of the black Aspergilli, mean that wines frequently contain low levels of ochratoxin A. Toxin production ceases at the time oxygen is removed from the must at the start of fermentation.

Where grapes are further processed to make dried vine fruits, the black Aspergilli continue to grow, as grapes are dried in the sun without preservatives in most producing countries. Dried grapes (raisins, sultanas) can therefore readily contain unacceptable levels of ochratoxin A (Lombaert et al., 2004; Iamanaka et al., 2005; Jorgensen, 2005; Meyvaci et al., 2005).

Many studies (e.g. Abarca et al., 2003 and many later papers) have now shown that *A. carbonarius* is the major source of ochratoxin A in wines and other grape products throughout the world. The incidence of fruit damage, grape splitting and high temperatures at harvest, together with poor harvest practice such as delayed crushing in wine making, all contribute to the level of ochratoxin A in wines and dried grape products.

The previous assessment (Annex 1, reference *153*) concluded that contamination of cereals with ochratoxin A occurs only in cool temperate zones, as the result of invasion by *P. verrucosum*. Although ochratoxin A was occasionally found in long-stored cereals, owing to the presence of *A. ochraceus* (or one of its close relatives), this is an uncommon occurrence (Pitt & Hocking, 1997). However, with the recent identification of *A. carbonarius* as a source of ochratoxin A in foods, the reported presence of ochratoxin A in tropical cereals from time to time can now be explained readily. It has been known for some years that *A. niger* commonly occurs in tropical cereals (Pitt et al., 1993, 1998), and all the evidence indicates that *A. carbonarius* will also be present under these conditions.

Aspergillus niger is by far the most common *Aspergillus* species responsible for post-harvest decay of fresh fruit, including apples, pears, peaches, citrus, grapes, figs, strawberries, mangoes, tomatoes and melons and some vegetables, especially onions, garlic and yams (Snowdon, 1990, 1991). It is not clear whether *A. carbonarius* occurs along with *A. niger* on these fresh foods. Most of these diseases are sporadic and of minor significance. Except where fruits are used for processing, rots due to *A. niger* are not considered a public health problem, as mouldy fruit is normally discarded.

Aspergillus niger is very frequently isolated from sun-dried products, such as vine fruits, dried, smoked and cured fish, biltong, cocoa beans and spices. It sometimes causes kernel rot in cashews and is one species causing thread mould spoilage of cheese (Hocking & Faedo, 1992).

Aspergillus niger is among the most common fungi isolated from nuts, especially peanuts, and has also been reported from pecans, pistachios, hazelnuts, walnuts, coconut and copra. Cereals and oilseeds are also sources, especially maize and also barley, soybeans, canola, sorghum, stored and parboiled rice and dried beans (Pitt & Hocking, 1997).

Aspergillus niger was commonly isolated during studies of Southeast Asian foods (Pitt et al., 1993, 1994, 1998). The highest contamination occurred in peanuts, maize, cashews, copra, pepper and spices from Indonesia, Thailand and the Philippines and in kemiri nuts from Indonesia.

In terms of mycotoxin production, *A. niger* is usually regarded as a benign fungus and has been widely used in food processing. It is categorized as "generally regarded as safe" by the government in the United States of America (USA). However, a low percentage (6%; Pardo et al., 2004) of *A. niger* isolates are believed to be able to produce ochratoxin A. Ochratoxin A production by *A. niger* in commercially grown crops appears possible, but is probably uncommon.

6. EFFECTS OF PROCESSING

A few recent studies have examined the effect of processing on concentrations of ochratoxin A in wines, coffee and European cereals.

6.1 Wines

Studies have shown that vinification consistently reduces ochratoxin A concentrations in wine, independent of the initial ochratoxin A concentration in grapes. Two independent studies produced ochratoxin in musts by inoculation of grapes on the vine with spores of *A. carbonarius.* In one study, the mean retention of ochratoxin A from grapes to wine was 8.1% after malolactic fermentation, without the use of fining agents (Fernandes et al., 2007). In a second study, the reduction of ochratoxin A during vinification was followed throughout the process, with the establishment of a mass balance for toxin loss (Leong et al., 2006a). Ochratoxin A was lost at each solid–liquid separation stage of the process. The ochratoxin A concentrations in white and red wines after racking were 4% and 9%, respectively, of those in crushed grapes. Those corresponded to 1% and 6% of the concentrations in the inoculated grapes (Leong et al., 2006a). The mass balance showed that ochratoxin A was not transformed either chemically or biologically by yeast during fermentation, but was discarded with the marc and lees. The greater retention of ochratoxin A in red wines was noted after two processes, pressing and racking. This was attributed to the fact that white grapes are pressed before fermentation, so (in the absence of alcohol) less ochratoxin A partitions into the juice; as a result, more is present in the grape solids and removed during clarification (Leong et al., 2006a). The addition of bentonite at 2.5 g/l to a semillon wine resulted in a 67% reduction in ochratoxin A concentration (Leong et al., 2006e). Addition of yeast hulls at 5.0 g/l caused a 43% reduction in ochratoxin A. Potassium caseinate and gelatin also had some effect on ochratoxin A concentrations, whereas a range of other fining agents had no effect. Storage of wines from 10 to 14 months resulted in decreases in ochratoxin A concentration of 22% and 29% for white and red wines, respectively (Leong et al., 2006d).

Another study examined the performance of 20 strains of *Saccharomyces* in removing naturally present ochratoxin A during vinification. Considerable strain-to-strain variation was observed: residual ochratoxin A ranged from 10% to 60%. When musts were fortified with additional ochratoxin A, residual ochratoxin A ranged from 17% to 34%. The authors concluded that the yeast cell walls were responsible for much of the reduction in ochratoxin A (Caridi et al., 2006).

All of these studies indicate that vinification results in large reductions of ochratoxin A during processing, although the percentage reduction varied considerably between the studies.

6.2 Coffee

A number of studies have looked at the influence of roasting on the ochratoxin A level in coffee. There is general agreement that roasting does have a big influence on ochratoxin A levels, but the percentage reduction reported has varied widely. The temperature of roasting appears to be the major factor influencing results. Using a roasting air temperature of 450 °C, three roasting times were studied by Romani et al. (2003) that resulted in final coffee temperatures of 175, 185 and 204 °C. The light roast (175 °C) caused no reduction in ochratoxin A in one sample, but reductions of 60–80% in three others. The dark roast (204 °C) caused

reductions of more than 90% in all samples. This process was equivalent to a typical espresso coffee brew.

Using artificially contaminated coffee beans (30 µg ochratoxin A/kg), Nehad et al. (2005) reported that roasting reduced ochratoxin A by 31%, that filtering reduced ochratoxin A by 72% and that preparation of Turkish coffee reduced ochratoxin A by 88%.

Pérez de Obanos et al. (2005) analysed nine samples of naturally contaminated Vietnamese coffee. Roasting for 5 min with air at 260 °C produced reductions ranging from 12% to 93%. Preparation of espresso coffee from these roasted samples produced a further reduction of 16–70% in ochratoxin A, whereas the production of mocha coffee caused reductions ranging from 17% to 55%, and the production of filtered coffee caused reductions that ranged from only 1.2% to 25%.

It can be concluded that coffee roasting reduces ochratoxin A in coffee, but the reductions are variable and not fully predictable.

6.3 Milling and breadmaking

Batches of whole wheat contaminated with ochratoxin A were produced by inoculation with *P. verrucosum* under controlled conditions in the laboratory. The fate of ochratoxin A was followed through cleaning, abrasive scouring of the outer grain coat, milling into wholemeal and milling into further fractions. Bread was baked from both wholemeal flour and straight-run white flour. Scouring removed up to 44% of the ochratoxin A present in the wheat, and only a small further loss occurred during the breadmaking process. Using a combination of cleaning, scouring and removal of the bran and offal fractions, an overall reduction of about 75% of ochratoxin A could be achieved in the white bread (Scudamore et al., 2003).

6.4 Extrusion

The extrusion of wholemeal wheat contaminated with ochratoxin A resulted in losses of no more than 40% of the toxin, even under the harshest conditions likely to be used in commercial practice (Scudamore et al., 2004; Castells et al., 2005). This reduction is much lower than had previously been reported for fumonisins, aflatoxins and zearalenone (Castells et al., 2005).

7. PREVENTION AND CONTROL OF OCHRATOXIN A PRODUCTION

The principal fungi that produce ochratoxin A in foods—*Aspergillus westerdijkiae* and *A. steynii* (formerly included in *A. ochraceus*), *A. carbonarius*, *Penicillium verrucosum* and *P. nordicum*—are not associated with plants and hence are not usually present in food crops before harvest. The control of ochratoxin A in foods, therefore, is basically a food technology problem. This means that the basic concepts of good harvest practice, of drying crops rapidly and keeping them dry in storage, transport and processing systems, will ensure that crops remain essentially free of ochratoxins (Pitt, 2006). There is one exception, and this is the possibility

of the entry of *A. carbonarius* into grapes before harvest. Each of the various commodity–fungus combinations will be dealt with in turn below, with the slightly more complicated situation of grapes first.

7.1 Grapes, wine and other grape products

Aspergillus carbonarius is not a pathogenic fungus in plants, and there is no evidence that it can invade healthy, intact grapes. However, various factors can cause damage to the skins of grapes before harvest, permitting the entry of this fungus. True grape pathogens, *Botrytis cinerea*, *Rhizopus stolonifer* and especially the powdery mildews, commonly infect grape bunches before harvest (Emmett et al., 1992; Leong et al., 2007). Effective control of these pathogens is very difficult, although reducing leaf numbers to increase sunlight on tight bunches and judicious spray programmes will help. In some cultivars, rain before harvest can cause skin splitting, and this also provides an entry point for *A. carbonarius* (Leong et al., 2004). Once *A. carbonarius* has gained entry to a grape, the high sugar/high acid combination provides a perfect medium for ochratoxin A production.

The keys to low ochratoxin A formation in wine, therefore, are the reliance on prevention of infection by pathogens, rapid harvest if rain causes grape splitting, good harvest practice, including rejection of poor quality bunches, and a minimal delay between harvest and crushing. Crushing rapidly leads to anaerobic conditions, where *A. carbonarius* can no longer grow and ochratoxin A formation ceases.

Control of ochratoxin A formation in dried vine fruits is less easy, because any preharvest infection with *A. carbonarius* will continue to develop during the early stages of drying. In addition, any damage to grapes during harvest and the commencement of drying can lead to new *A. carbonarius* infections. Although the hot, high sunlight levels that occur during drying are not favourable to the growth of *A. carbonarius*, this fungus is less inhibited than almost any other species, and so ochratoxin A production can continue until the water activity of the drying grapes has been reduced substantially (Leong et al., 2006c).

7.2 Coffee

Aspergillus westerdijkiae and *A. steynii* (formerly included in *A. ochraceus*) and *A. carbonarius* are the major causes of ochratoxin production in green coffee. There is no evidence that these fungi invade coffee cherries before harvest. Infection occurs during handling after harvest and in the drying yard (Taniwaki et al., 2003).

Coffee plants originated in the highlands of Ethiopia and must be exposed to temperatures below 19 °C in order for flowering to occur. However, berry maturation requires much higher temperatures, so world coffee production occurs in tropical highland regions. In consequence, many coffee-growing areas in the world are subjected to misty or rainy conditions after harvest, with consequent slow drying of the coffee cherries, providing the time needed for these fungi to invade and produce ochratoxin A. However, good processing and drying regimes can prevent this problem. It has been shown that rapid and efficient drying of coffee

cherries after harvest leads to the production of coffee beans free of ochratoxin A (Bucheli & Taniwaki, 2002).

The theory of producing coffee free of ochratoxin is clear, but it will be some time before practical measures can be put in place in all growing areas.

7.3 Cereals in temperate zones

Ochratoxin A in temperate zone cereals is produced by the growth of *P. verrucosum.* Again, there is no evidence that these fungi infect growing cereal plants or occur in nature in grains before harvest. The key to controlling ochratoxin A in cereals is again good drying practice. The theory is clear; however, in cool temperate zones, grain is often harvested during moist or rainy conditions, and control may be difficult in practice. A European Union (EU) project recently examined all aspects of this problem and issued a major report (Olsen et al., 2004).

7.4 Cereals in tropical zones

The crop most at risk from ochratoxin A production in the tropics is maize, as the black Aspergilli are commonly present in maize grains, but much less common in other cereals (Pitt et al., 1993, 1994, 1998). Ochratoxin production in maize was not considered to be a problem until quite recently, and in general terms it is much less significant than production of aflatoxins or fumonisins. It is most likely that *A. carbonarius* is the fungus responsible for ochratoxin production in tropical crops such as maize, but this topic has been little researched. *Aspergillus carbonarius* was not distinguished from *A. niger* by Pitt et al. (1993, 1994, 1998). Good drying and storage practice should lead to control of ochratoxin in tropical crops such as maize.

7.5 Meat products

If meat products are infected by *P. nordicum*, this must occur during the processing stages. Control of levels of fungal spores in the air will reduce this problem.

8. FOOD CONSUMPTION AND DIETARY EXPOSURE ASSESSMENTS

The request from CCFAC (Codex Alimentarius Commission, 2006) was to review the available data on the contamination of cereals by ochratoxin A with a particular focus on cereals from developing countries and to assess the possible impact of MLs of 5 and 20 µg/kg for cereals. In addition, the potential impact of new data on the previous exposure assessment done by the Committee was estimated.

8.1 Analysis of data submitted

Data on the occurrence of ochratoxin A in cereals were submitted by Canada, Germany, Japan and the EU. In addition, data reporting the contamination of cereals in Africa (Nigeria, Ghana and Burkina Faso) were submitted jointly by the

Food and Agriculture Organization of the United Nations (FAO) and Consiglio Nationale delle Ricerche (CNR), Italy, and information regarding the contamination of cocoa and coffee beans was submitted by Côte d'Ivoire. Table 8 describes the various aspects of the distribution of ochratoxin A contamination in cereals.

8.1.1 Canada

Canada submitted a probabilistic exposure assessment based on occurrence data for ochratoxin A in cereals and other food commodities and on food consumption data from the USA. Detailed individual data on occurrence were not submitted, but ochratoxin A was detected in 436 samples out of 1668 analytical results.

Table 8. Description of data submitted for this evaluation: distribution of ochratoxin A contamination in cereals

Countries	Number of samples	Number of non-detected[a] (%)	Samples >LOD or LOQ and <5 µg/kg (%)	Samples ≥5 µg/kg (%)	Samples >10 µg/kg (%)	Samples >15 µg/kg (%)	Samples >20 µg/kg (%)
Germany (RC)	1719	1428 (83)	250 (15)	18 (1.1)	8 (0.5)	6 (0.4)	6 (0.4)
Germany (PC)	2377	1717 (72)	639 (27)	21 (0.9)	11 (0.5)	5 (0.2)	2 (0.1)
Japan	197	188 (95)	9 (5)	0	0	0	0
Africa	27	9 (33)	16 (59)	2	0	0	0
Canada (RC)	1016	827 (82)	–	–	–	–	–
Canada (PC)	652	405 (62)	–	–	–	–	–
EU (RC + PC)	5117	2295 (45)	2779 (54)	43 (0.8)	17 (0.3)	–	10 (0.2)

LOD, limit of detection; LOQ, limit of quantification; RC, raw cereals; PC, processed cereals.
[a] Non-detected samples are those for which no numerical value was provided. They can be either results below the LOD or in certain cases results between the LOD and the LOQ.

The report provides various estimations of the average contamination level, replacing the left-censored data by half of the limit of detection (LOD) or half of the limit of quantification (LOQ) when the LOD was not provided (scenario 1) or applying a parametric model assuming the lognormality of the distribution (scenario 2). The authors also truncated the distribution assuming that an ML was implemented and fully enforced (scenario 3) and compared the resulting mean concentration with a distribution not censored on the right (scenario 4). Interestingly, none of the scenarios shows a significant impact on the average concentration for each lot analysed. However, the observed differences in the mean contaminations are

obviously driven by the LOD and LOQ, because for samples analysed by Health Canada, the LOD was 0.05 µg/kg for most of the cereals, whereas for the data analysed by the Canadian Grain Commission, the LOD was not available and an LOQ of 1 µg/kg was used to calculate the average contamination level.

For the assessment of dietary exposure, the authors used the full distribution of the contamination for all commodities combined with the frequencies and amounts consumed and estimated an average dietary exposure below 5 ng/kg bw and high percentiles of exposure below 15 ng/kg bw. Because of the methodological choice of a probabilistic exposure assessment, cut-off points on the right of the distribution curve to simulate enforced MLs have an impact on dietary exposure when the cut-off points are located in the tail (e.g. at the level of 5 µg/kg). On the contrary, a cut-off point at a level above most of the observed values of contamination (e.g. 20 µg/kg) is ineffective.

8.1.2 Germany

Germany submitted 4097 individual analytical results for ochratoxin A collected between 1999 and 2006 in different cereals and foodstuffs derived from cereals. Data are described in Table 8.

The assays were distributed as follows:

- 624 samples of wheat and 580 samples of wheat flour and other wheat products;
- 414 samples of rye and 368 samples of rye flour and other rye products;
- 197 samples of barley and 16 samples of barley products;
- 30 samples of oat and 249 samples of oat flakes and other oat products;
- 19 samples of maize and 221 samples of maize meal, cornflakes and other maize products;
- 285 samples of rice and 21 samples of rice products;
- 30 samples of buckwheat and 18 samples of buckwheat products;
- 499 samples of muesli and muesli products;
- 120 samples of other cereals and 405 samples of products derived from other cereals.

The highest ochratoxin A levels in unprocessed cereals were in rye and buckwheat. Three samples of rye contained a very high concentration of ochratoxin A (95.6–125 µg/kg). A high number (33.3%) of analysed samples of oat contain elevated levels (mean = 0.27 µg/kg).

In those products derived from cereals, the highest ochratoxin A levels were found in products derived from buckwheat (mean = 1.94 µg/kg) and from rye (mean = 0.54 µg/kg).

8.1.3 Japan

Japan submitted individual analytical data for 98 samples of rice and 99 samples of wheat. For rice, only one sample contained ochratoxin A above the LOD (0.08 µg/kg) but below the LOQ. For wheat, seven samples were above the LOD

(0.07 µg/kg) and below the LOQ, and only one sample was above the LOQ, with an ochratoxin A concentration of 0.7 µg/kg.

8.1.4 European Union

The European Commission submitted a report compiling 5117 (aggregated) analytical results for ochratoxin A in cereals and cereal products, collected by EU Member States (European Commission, 2002). The total number of positive samples for cereals and cereal products was 55%, and they ranged in concentration from the LOD (0.005 µg/kg) to 33.3 µg/kg. The weighted mean (assuming that the samples in which ochratoxin A was not detected [ND] are contaminated at half of the LOD) was 0.29 µg/kg.

Since information on the extent of contamination in raw materials is very relevant, not only from the health perspective, but also from the economic and commercial points of view, the occurrence data for cereal raw materials (wheat, corn, oat, millet, rye, barley and rice) were reported separately. The following can be noted:

- The weighted mean (assuming that the samples in which ochratoxin A was ND are contaminated at half of the LOD) ranged from 0.136 µg/kg for millet (n = 34) to 0.597 µg/kg for rye (n = 444).
- Among the individual cereal commodities, wheat has been investigated more widely than the other grains, especially in northern Europe.
- Rye contained the highest ochratoxin A levels, with 50% positive samples.

8.1.5 Nigeria, Ghana and Burkina Faso

CNR, in agreement with FAO, provided 27 analytical results on ochratoxin A contamination of cereals in Nigeria, Ghana and Burkina Faso. Ochratoxin A was detected in sorghum, maize and millet samples. Maize had the highest levels of ochratoxin A contamination.

8.2 Estimation of the content of ochratoxin A in cereals

8.2.1 Background

The critical effects of ochratoxin A relate to long-term exposure. Furthermore, considering that the variability of ochratoxin A concentrations in food depends on climatic and storage conditions, it is unlikely that most consumers would be exposed on a long-term basis to high levels of contamination. Therefore, the central tendency of the distribution of contamination should be combined with food consumption levels for dietary exposure assessments. In addition, owing to the fact that generally more than 50% of analysed samples are below the LOD or LOQ, the use of the median value to represent this central tendency is problematic. Therefore, the international exposure assessments to date are based on the mean level of contamination.

In 2001, the Committee estimated the overall dietary exposure of ochratoxin A to be 43 ng/kg bw per week. The contribution from cereals was estimated to be 58% of the overall exposure (25 ng/kg bw per week), and the level of exposure from food sources other than cereals was therefore equal to 42% of the overall exposure, corresponding to 18 ng/kg bw per week.

In 2006, EFSA evaluated ochratoxin A and estimated a total mean dietary exposure of 15–20 ng/kg bw per week with a high percentile around 60 ng/kg bw per week, based on an average contamination for cereals of 0.29 µg/kg and a high percentile for cereal consumption of 568 g/day (97.5th percentile consumers only in Sweden) (European Food Safety Authority, 2006).

8.2.2 Estimation of the mean contamination for ochratoxin A

African samples were not used for the dietary exposure assessment because they were targeted samples with fungal contamination. Individual data were not available either from Canada or from the EU. Finally, the Committee decided not to merge individual analytical data from Germany and Japan and to focus for the dietary exposure on the largest data set represented by German analytical results for raw and processed cereals.

In order to estimate the mean contamination of ochratoxin A in cereals, a strategy must be applied regarding the replacement of values for samples below the LOD/LOQ. Several approaches can be used, either by replacing the value by 0, LOD/2 or LOD or by modelling the distribution (Verger & Tressou, 2006). For the purpose of the current assessment and considering that more than 60% of samples are not detected, the assignment of ND equal to 0 and ND equal to LOD or LOQ was used to provide a lower- and an upper-bound concentration of ochratoxin A in cereals and to be consistent with World Health Organization (WHO) recommendations.

Analytical results (detected or non-detected) for which information on LOD or LOQ was not available were removed from the data set. In addition, analytical results obtained with methods with high LOQs (i.e. higher than the mean concentration) can artificially influence the estimation of the mean contamination. Therefore, the impact of removing analytical data obtained with the LOQs between 0.5 and up to 3 µg/kg was tested. The Committee concluded that analytical results with an LOQ higher than 1 µg/kg were not fit for the purpose of the current assessment and were removed from the data set. The Committee also considered that removing results obtained with an LOQ between 0.5 and 1 µg/kg would not result in additional improvement of the estimate.

Finally, 1462 and 2070 analytical results were used, respectively, for raw cereals and cereal products. The resulting mean and other main statistical descriptors of the distribution curves are compiled in Tables 9 and 10.

Table 9. Ochratoxin A distribution in the German data set on raw cereals (1462 samples): impact of various MLs on the mean concentration of ochratoxin A

	Concentration of ochratoxin A in raw cereals (µg/kg)	
	ND = 0	ND = LOD or LOQ
Distribution of ochratoxin A		
Mean	0.44	0.53
Median	0.00	0.10
90th percentile	0.26	0.50
95th percentile	0.78	0.90
99th percentile	4.98	4.98
Maximum	125.00	125.00
Impact of MLs on mean concentration of ochratoxin A		
ML = 5 µg/kg	0.10	0.19
ML = 20 µg/kg	0.15	0.24
No ML	0.44	0.53

Table 10. Description of ochratoxin A distribution in the German data set on processed cereals (2070 samples): impact of various MLs on the mean concentration of ochratoxin A

	Concentration of ochratoxin A in processed cereals (µg/kg)	
Distribution of ochratoxin A	ND = 0	ND = LOD or LOQ
Mean	0.31	0.39
Median	0.00	0.10
90th percentile	0.65	0.70
95th percentile	1.40	1.40
99th percentile	4.73	4.73
Maximum	22.80	22.80

8.2.3 Impact of new data submitted on estimate of mean ochratoxin A concentration

Based on the submission of more than 11 000 samples of cereals and cereal products (individual + aggregated), only 87 of them (0.8%) are contaminated with ochratoxin A at concentrations between 5 and 20 µg/kg, and only 21 samples are contaminated above 20 µg/kg (0.2%); therefore, the theoretical difference between the two MLs of 5 and 20 µg/kg would represent about 0.6% of cereals.

From the German data described above and in Table 9, it is possible to simulate the impact of setting various MLs, assuming 100% enforcement, on mean ochratoxin A concentration by excluding analytical results above each ML. Based

on the available individual analytical results for raw cereals, the mean contamination was calculated using two scenarios for left censorship (i.e. ND = 0 and ND = LOD/LOQ), two scenarios for right censorship (i.e. ML at 5 or 20 µg/kg) and finally a scenario without an ML, resulting in six scenarios compiled in Table 9.

The available data do show a limited impact of setting any of the proposed MLs on the mean contamination of raw cereals by ochratoxin A. However, it is important to note that the observed differences are due to very few analytical results (15 samples out of 1462 excluded when the ML is 5 µg/kg and 6 samples out of 1462 excluded when the ML is 20 µg/kg).

8.2.4 Impact of new data submitted on estimate of dietary exposure

In 2001, the mean overall dietary exposure to ochratoxin A based mainly on European data was estimated by the Committee to be 43 ng/kg bw per week. The contribution from cereals was estimated to be 58% of the overall exposure (25 ng/kg bw per week) based on a daily consumption of 230 g of cereals and a mean concentration of ochratoxin A of 0.94 µg/kg for raw cereals.

The concentrations of ochratoxin A in processed cereals submitted to this Committee are detailed in Table 10. The mean concentration for ochratoxin A ranges from 0.31 µg/kg (lower-bound estimate with assignment of 0 for ND samples) to 0.39 µg/kg (upper-bound estimate with assignment of LOD or LOQ for ND samples). The Committee decided to consider both the mean consumption of cereals from the five Global Environment Monitoring System (GEMS) regional diets (230 g in Europe) and the mean consumption of cereals for Germany from the 13 GEMS Food Contamination Monitoring and Assessment Programme (GEMS/Food) Consumption Cluster Diets (365 g, cluster E). The resulting dietary exposure estimate ranged from 8 to 17 ng/kg bw per week, which can be compared with the previous estimate of 25 ng/kg bw per week.

9. COMMENTS

9.1 Absorption, distribution, metabolism and excretion

Ochratoxin A is efficiently absorbed from the gastrointestinal tract, mainly in the small intestine. Information from a number of species shows that it is distributed via the blood mainly to the kidneys, with lower concentrations found in liver, muscle and fat. Specific transporters may be involved in the cellular uptake of ochratoxin A into the kidney, where it accumulates. Transfer to milk has been demonstrated in rats, rabbits and humans, but little ochratoxin A is transferred to the milk of ruminants, owing to efficient hydrolysis of the amide bond by microflora in the rumen, to yield phenylalanine and ochratoxin *alpha*. Ochratoxin *alpha*, a chlorinated dihydroisocoumarin, is the major metabolite of ochratoxin A in all species examined. This and minor hydroxylated metabolites of ochratoxin A that have been identified are all reported to be less toxic than ochratoxin A itself. Ochratoxin A is excreted in urine and faeces, and the relative contribution of each of these routes in different species is influenced by the extent of enterohepatic recirculation of ochratoxin A

and its binding to serum proteins. These factors are also important in the determination of the serum half-life of ochratoxin A, which varies widely among species. Ochratoxin A has a long half-life in some non-ruminant mammals, ranging from 1–1.5 days in mice, 2–5 days in rats and 3–5 days in pigs up to around 20 days in macaque and vervet monkeys and 35 days in a human volunteer.

9.2 Toxicological data

None of the new studies on nephrotoxicity, developmental toxicity, neurotoxicity or immunotoxicity that have appeared since the Committee's last evaluation would have an impact on the Committee's previous selection of minimal renal changes in the pig, observed at a dose of 8 µg/kg bw per day (the LOEL), as a critical effect for risk assessment.

In its previous evaluation, the Committee commented that the mechanism by which ochratoxin A causes renal tumours was unknown, noting that both genotoxic and non-genotoxic (epigenetic) modes of action had been proposed. Investigation of the mode of action of ochratoxin A in the kidney, with particular reference to carcinogenic effects, has been a key driver of much of the research conducted since then.

Several hypotheses on the mode of action of ochratoxin A as a carcinogen have been proposed, and evidence has been generated in support of each of them. Some of these would completely account for tumour formation, whereas others have been considered as possible contributors to tumour formation. They can be summarized as follows:

- genotoxicity from direct interaction of ochratoxin A or a reactive metabolite with DNA;
- generation of tumours secondary to chronic renal toxicity and compensatory cell proliferation;
- generation of tumours secondary to inhibition of phenylalanine–$tRNA^{Phe}$ synthetase and protein synthesis;
- disruption of cell–cell signalling pathways and the process of cell division;
- alteration of intracellular calcium homeostasis;
- mitochondrial dysfunction leading to oxidative stress and indirect induction of DNA damage.

Concerning a genotoxic mode of action, divergent results have been obtained in the large number of genotoxicity assays on ochratoxin A, most of which were available at the time of the Committee's previous evaluation. In more recent comet assays, there was evidence of DNA damage in vitro and in vivo, including in rat kidney. However, these positive results were generally obtained with high ochratoxin A exposure levels and, where investigated, were indicative of oxidative damage. The Committee noted that whereas some investigators have previously reported formation of a number of different DNA adducts detectable by the ^{32}P-postlabelling technique under different in vitro and in vivo conditions, particularly following prolonged, high exposures to ochratoxin A, others have not been able to detect DNA adduct formation, despite, in some cases, using similar doses of

ochratoxin A and more sensitive techniques. The Committee concluded that a direct genotoxic mode of action, by demonstration of covalent binding to DNA with the formation of DNA adducts containing ochratoxin A or a metabolite of ochratoxin A, has not been confirmed.

Concerning non-genotoxic modes of action, a number of recent studies have addressed early changes associated with ochratoxin A exposure in vitro and in vivo, including indicators of oxidative stress, alterations in gene expression and cell signalling pathways, increased apoptosis, disruption of cell mitosis and increases in cell proliferation. Recent work has shown marked dose- and time-related increases in renal cell proliferation after 4 and 13 weeks of gavage administration of ochratoxin A to rats at the same doses that gave rise to renal tumour formation in the 2-year rat study (70 and 210 µg/kg bw per day, 5 days/week), with a NOAEL for cell proliferation at the same dose as that where no increase in tumour formation was observed in the 2-year rat study (21 µg/kg bw per day, 5 days/week). The Committee noted that cell proliferation, which is known to be effective in increasing tumour incidence via conversion of DNA damage into permanent mutations, may be a key event in the mechanism of tumour formation with ochratoxin A. Overall, the Committee considered that the evidence points to a number of non-genotoxic modes of action that could plausibly be involved in the generation of renal tumours, and this supports the previous decision to set a PTWI.

In order to provide additional information for the risk assessment, the Committee performed BMD modelling using the carcinogenicity data on ochratoxin A from the rat bioassay performed by the NTP in 1989 (National Toxicology Program, 1989). The combined adenoma and carcinoma data from male rat kidney, representing the most sensitive sex, species and target organ for ochratoxin A carcinogenicity, were used for modelling. Six different models were fitted to the dose–incidence data to estimate the lower limits of one-sided 95% confidence intervals on the BMD representing a 10% renal tumour incidence ($BMDL_{10}$s). The lowest $BMDL_{10}$ had a value of 15 µg/kg bw per day, 5 days/week, and the model showing the best fit had a value of 25 µg/kg bw per day, 5 days/week. Thus, for establishing the PTWI, the $BMDL_{10}$ does not provide a lower point of departure than the LOEL of 8 µg/kg bw per day for minimal renal toxicity changes in the pig.

9.3 Observations in humans

The earlier literature on the association between human exposure to ochratoxin A and the occurrence of Balkan endemic nephropathy and associated urinary tract tumours was summarized in the previous evaluation (Annex 1, reference *153*). Contrary to the clear causal evidence of ochratoxin A–induced nephrotoxicity and kidney carcinogenicity in rodents, the significance of ochratoxin A for human health remains unclear from the available epidemiological evidence. Moreover, ochratoxin A exposure is only one of several hypotheses concerning an environmental etiology for Balkan endemic nephropathy.

Blood concentration of ochratoxin A appears to be a reliable biomarker of exposure in humans. In the Committee's previous evaluation (Annex 1, reference *153*), the concentrations of ochratoxin A in blood samples from healthy persons,

obtained in surveys conducted in 17, mainly European, countries, ranged between 0.1 and 40 ng/ml (with an exceptional maximum of 160 ng/ml). The concentrations of ochratoxin A in blood samples obtained in more recent surveys from nine countries, four of which are European, ranged between 0.15 and 1.14 ng/ml, suggesting a possible decline in extreme peak values for blood concentrations compared with earlier surveys.

9.4 Analytical methods

Methods for analysing ochratoxin A were thoroughly reviewed in the previous report (Annex 1, reference *153*). At that time, validated analytical methods were already available for the determination of ochratoxin A in maize, barley, rye, wheat, wheat bran, wholemeal wheat, roasted coffee, wine and beer. The best methods used LC following cleanup using immunoaffinity columns. Recent developments in analytical methodology are described in section 3 of this monograph.

9.5 Sampling protocols

The only recent development in this area has been the publication of extensive information on the parameters governing sample size for testing green coffee for ochratoxin A.

9.6 Fungi producing ochratoxin A

At the fifty-sixth meeting of JECFA (Annex 1, reference *152*), it was reported that ochratoxin A is produced by three taxonomically distinct groups of fungi: a single *Penicillium* species, *P. verrucosum*; *Aspergillus ochraceus* and several related *Aspergillus* species; and *A. carbonarius*, with a small percentage of isolates of the closely related species *A. niger.* Since that report, some other *Aspergillus* and *Penicillium* species have been described as potential sources of ochratoxin A. Details of the revised taxonomy of the fungi producing ochratoxin A and of the physiology and ecology of these fungi are described in section 5.1 of this monograph.

9.7 Effects of processing

A few recent studies have examined the effect of processing on concentrations of ochratoxin A in wine, coffee and European cereals.

9.7.1 Wine

Vinification has consistently been reported to reduce ochratoxin A concentrations in wine, independent of the initial ochratoxin A concentration in grapes. Ochratoxin A is removed at each solid–liquid separation stage of the process.

9.7.2 Coffee

There is general agreement that roasting has an influence on ochratoxin A concentrations, but the percentage reduction reported has varied widely. Light

roasting causes reductions in ochratoxin A of 0–80%. Dark roasting (i.e. to a typical espresso coffee) may cause reductions of more than 90% in ochratoxin A content.

9.7.3 Milling and breadmaking

Using whole wheat contaminated with ochratoxin A, a combination of cleaning, scouring, removal of the bran fraction and baking caused an overall reduction of about 75% of ochratoxin A in white bread.

9.7.4 Extrusion

The extrusion of wholemeal wheat contaminated with ochratoxin A resulted in a reduction of no more than 40% of the toxin, even under the harshest conditions likely to be used in commercial practice.

9.8 Prevention and control

The principal fungi that produce ochratoxin A in foods—*Aspergillus carbonarius*, *A. westerdijkiae*, *A. steynii*, *Penicillium verrucosum* and *P. nordicum*—are not associated with plants and hence are not usually present in food crops before harvest. The control of ochratoxin A in foods, therefore, is basically a post-harvest problem. The basic concepts of good harvest practice, of drying crops rapidly and keeping them dry in storage, transport and processing systems, will ensure that crops remain essentially free of ochratoxin A. One exception is the entry of *A. carbonarius* into grapes before harvest.

9.8.1 Grapes, wine and other grape products

Once *A. carbonarius* has gained entry to a grape via damaged skin, the high sugar/high acid combination provides a perfect medium for ochratoxin A production. The keys to low ochratoxin A levels in wine are the reliance on prevention of infection of grapes by pathogens, rapid harvest if rain causes skin splitting, good harvest practice, including rejection of poor quality bunches, and a minimal delay between harvest and crushing. Control of ochratoxin A formation in dried vine fruits is less easy, because any pre-harvest infection with *A. carbonarius* will continue to develop during the early stages of drying.

9.8.2 Coffee

Aspergillus westerdijkiae, *A. steynii* and *A. carbonarius* are the major causes of ochratoxin production in green coffee. Infection of coffee cherries occurs during handling after harvest and in the drying yard. Many coffee-growing areas in the world are subjected to misty or rainy conditions after harvest, with consequent slow drying of the coffee cherries and ochratoxin A formation. However, good processing and drying regimes can prevent this problem.

9.8.3 Cereals

Where ochratoxin A occurs in cool temperate zone cereals, it is produced by the growth of *P. verrucosum.* Again, there is no evidence that this species infects

growing cereal plants or occurs in nature in grains before harvest. The key to controlling ochratoxin A in cereals is rapid drying; however, in cool temperate zones, grain is often harvested during moist or rainy conditions, and rapid drying may be difficult in practice.

There were limited data available to the Committee on cereals grown in tropical zones, but ochratoxin A has been found to occur in sorghum, maize and millet.

9.8.4 Meat products

If meat products are infected by *P. nordicum*, this occurs during the processing stages. Control of levels of fungal spores in the air will reduce this problem.

9.9 Dietary exposure assessment

9.9.1 Analysis of data submitted

Data on occurrence of ochratoxin A in cereals were submitted by Canada, Germany, Japan and the EU. In addition, data reporting the contamination of cereals in Nigeria, Ghana and Burkina Faso were submitted by FAO, and information regarding the contamination of cocoa and coffee beans was submitted by Côte d'Ivoire. Table 8 in section 8.1 describes the various aspects of the distribution of ochratoxin A contamination in cereals. The most contaminated commodities in the German data set were rye and buckwheat, whereas the most contaminated commodities in the African data set were sorghum, maize and milletons.

9.9.2 Estimation of the concentration of ochratoxin A in cereals

The critical effects of ochratoxin A relate to long-term exposure, and therefore the central tendency of the distribution of contamination should be used for dietary exposure assessments. Owing to the fact that generally more than 50% of analysed samples are below the LOD or LOQ, the use of the median value to represent this central tendency is problematic. Therefore, the international exposure assessments to date are based on the mean level of contamination.

The data from Africa were not used for the dietary exposure assessment because they were targeted samples with fungal contamination. Individual data were not available from either Canada or the EU. Finally, the Committee decided not to merge individual analytical data from Germany and Japan, but to focus the dietary exposure assessment on the largest data set of analytical results for raw and processed cereals from Germany.

9.9.3 Impact of left and right censorship on average level of contamination

Information on LOD and LOQ is essential for the purpose of the current assessment to assign values to ND results. The assignment of ND equal to 0 and ND equal to LOD or LOQ was used to provide a lower- and an upper-bound concentration of ochratoxin A in cereals.

Analytical results (detected or non-detected) for which information on LOD or LOQ was not available were removed from the data set. In addition, because available LOD or LOQ values were very variable, the impact of removing analytical data obtained with the highest LOQs was tested. The Committee excluded all samples (detected and non-detected) where the LOQ was higher than 1 µg/kg because the method of analysis was not very sensitive and there were sufficient data for the analysis obtained by more sensitive methods.

From the available data, it is possible to simulate the impact of various MLs, assuming 100% enforcement, on mean ochratoxin A concentration by excluding analytical results above each ML. Based on the available individual analytical results for raw cereals from Germany, the mean contamination was calculated using two scenarios for left censorship (i.e. ND = 0 and ND = LOD or LOQ), two scenarios for right censorship (i.e. ML at 5 or 20 µg/kg) and finally a scenario without an ML, resulting in six scenarios compiled in Table 9 in section 8.2.2.

The available data show a limited impact of the various MLs on the mean contamination of raw cereals by ochratoxin A. However, it is important to note that the observed differences are due to very few high analytical results (15 samples out of 1462 excluded when the ML is 5 µg/kg and 6 samples out of 1462 excluded when the ML is 20 µg/kg).

9.9.4 Impact of new data on estimates of dietary exposure to ochratoxin A

In 2001, the mean overall dietary exposure to ochratoxin A, based mainly on European data, was estimated by the Committee to be 43 ng/kg bw per week (Annex 1, reference *152*). The contribution from cereals was estimated to be 58% of the overall exposure (25 ng/kg bw per week), based on a daily consumption of 230 g of cereals and a mean ochratoxin A concentration of 0.94 µg/kg for raw cereals.

At the present meeting, mean concentrations of ochratoxin A for processed cereals were estimated to be 0.31 µg/kg for the lower bound and 0.39 µg/kg for the upper bound, based on German data (Table 10 in section 8.2.2). In order to perform a realistic estimate of dietary exposure from the consumption of cereals, the Committee decided to consider both the mean consumption of cereals from the five GEMS regional diets (230 g in Europe) and the mean consumption of cereals from the 13 GEMS/Food Consumption Cluster Diets (365 g in cluster E, includes Germany). The resulting dietary exposure estimate ranged from 8 to 17 ng/kg bw per week, which can be compared with the previous estimate of 25 ng/kg bw per week.

10. EVALUATION

The new data, including data on mode of action of ochratoxin A in the kidney, do not indicate any reason to modify the previous approach taken by JECFA with respect to setting a PTWI. The Committee therefore retained the previous PTWI of 100 ng/kg bw.

The current estimate of overall dietary exposure to ochratoxin A from cereals, based mainly on European data, is about 8–17 ng/kg bw per week, based on processed cereals, compared with 25 ng/kg bw per week in the previous evaluation, based on raw cereals. The current estimate is well below the PTWI.

Contamination levels in the majority of raw cereal samples were below 5 µg/kg. Owing to the very small number of samples contaminated above the highest proposed limit of 20 µg/kg for cereals, such an ML would have very limited impact compared with no ML. The Committee concluded that the use of an ML of 5 or 20 µg/kg would be unlikely to have an impact on dietary exposure to ochratoxin A. The Committee was unable to reach a conclusion regarding the situation in developing countries, owing to the lack of adequate data with which to do an assessment.

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ANNEXES

ANNEX 1

REPORTS AND OTHER DOCUMENTS RESULTING FROM PREVIOUS MEETINGS OF THE JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

1. *General principles governing the use of food additives* (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. *Procedures for the testing of intentional food additives to establish their safety for use* (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. *Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants)* (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, Vol. I. *Antimicrobial preservatives and antioxidants*, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. *Specifications for identity and purity of food additives (food colours)* (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as *Specifications for identity and purity of food additives*, Vol. II. *Food colours*, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. *Evaluation of the carcinogenic hazards of food additives* (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. *Evaluation of the toxicity of a number of antimicrobials and antioxidants* (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. *Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents* (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. *Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants* (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. *Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants*. FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. *Specifications for identity and purity and toxicological evaluation of food colours*. FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.
11. *Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases* (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).
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ANNEX 2

ABBREVIATIONS USED IN THE MONOGRAPHS

6-GT	6-α-glucosyltransferase
αAsGM1	anti-asialoGM_1
ADI	acceptable daily intake
AFB_1	aflatoxin B_1
AFB_2	aflatoxin B_2
AFC	antibody forming cells
AFG_1	aflatoxin G_1
AFG_2	aflatoxin G_2
AFL	aflatoxin
AFT	total aflatoxin
AIC	Akaike information criterion
ALT	alanine aminotransferase
ANOVA	analysis of variance
ASC	acidified sodium chlorite
ASNU	asparaginase units
ATP	adenosine triphosphate
AUC	area under the curve
a_w	water activity
BCRP	breast cancer resistance protein
BMD	benchmark dose
BMD_{10}	BMD for a 10% increase in effect of concern
BMDL	95% lower confidence limit of the BMD
$BMDL_{10}$	95% lower confidence limit of the BMD_{10}
BUN	blood urea nitrogen
bw	body weight
CAS	Chemical Abstracts Service
CC	column chromatography
CCFAC	Codex Committee on Food Additives and Contaminants
cDNA	complementary deoxyribonucleic acid
CGTase	cyclodextrin glycosyltransferase
CHO	Chinese hamster ovary
CI	confidence interval
CNR	Consiglio Nationale delle Ricerche (Italy)
cpm	counts per minute
CSFII	Continuing Survey of Food Intakes by Individuals (USA)
CV	coefficient of variation
Cx	connexin
CYP	cytochrome P450
Da	dalton
dG	deoxyguanosine
dGuoOTA	ochratoxin A–deoxyguanosine adduct
DMBA	dimethylbenzanthracene

DNA	deoxyribonucleic acid
EC	European Commission
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
Endo III	endonuclease III
ERK 1/2	extracellular signal-regulated kinase 1 and 2
EU	European Union
F	female
FAO	Food and Agriculture Organization of the United Nations
FLD	fluorescence detector
FOB	functional observational battery
Fpg	formamido-pyrimidine-DNA-glycosylase
G6PD	glucose-6-phosphate dehydrogenase
GD	gestation day
GEMS	Global Environment Monitoring System
GEMS/Food	Global Environment Monitoring System Food Contamination Monitoring and Assessment Programme
GJIC	gap junction intercellular communication
GLP	Good Laboratory Practice
GSH	glutathione
GST	glutathione *S*-transferase
$HBsAg^+$	hepatitis B virus surface antigen positive
$HBsAg^-$	hepatitis B virus surface antigen negative
HDAC	histone deacetylase
HDL	high-density lipoprotein
HLA	human histocompatibility leukocyte antigen
HNF4α	hepatocyte nuclear factor 4-alpha
HPLC	high-performance liquid chromatography
HPV	human papillomavirus
IAC	immunoaffinity column
IAU	isoamylase units
IC_{50}	median inhibitory concentration
IFN	interferon
Ig	immunoglobulin
IGF-1r	insulin-like growth factor-1 receptor
IL	interleukin
IMT	α-isomaltosyltransferase
IPCS	International Programme on Chemical Safety (WHO)
IU	international units
IUCLID	International Uniform Chemical Information Database
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JNK	c-jun *N*-terminal kinase
LC	liquid chromatography
LD_{50}	median lethal dose

LDH	lactate dehydrogenase
LDL	low-density lipoprotein
LEC	liquid enzyme concentrate
LEU	lecitase units
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOEL	lowest-observed-effect level
LOQ	limit of quantification
LOR	limit of reporting
LPO	lipid peroxidation
M	male
MAPK	mitogen-activated protein kinases
MDCK	Madin-Darby canine kidney
MDR1	Multidrug Resistance 1
ML	maximum limit
MNU	*N*-methyl-*N*-nitrosourea
mRNA	messenger ribonucleic acid
MS/MS	tandem mass spectrometry
NA	not available
NAD^+	nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ND	no data reported; not detected
NF	nuclear factor
NF-κB	nuclear factor-kappa B
NK	natural killer
NNS	National Nutrition Survey (Australia/New Zealand)
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NR	not reported
Nrf2	nuclear factor-erythroid 2 p45-related factor
NTP	National Toxicology Program (USA)
OAT	organic anion transporter
OECD	Organisation for Economic Co-operation and Development
OK	opossum kidney
OR	odds ratio
OTA-3′-dGMP	ochratoxin A-3′-monophosphate-deoxyguanosine
OTHQ	ochratoxin hydroquinone
OTQ	ochratoxin quinone
p38	protein kinase 38
PC	processed cereals
PDK1	phosphoinositide-dependent kinase-1
PKC	protein kinase C
PMTDI	provisional maximum tolerable daily intake
PND	postnatal day

PRK	primary rat kidney
Px	xth percentile
PTWI	provisional tolerable weekly intake
QA	quality assurance
QC	quality control
RC	raw cereals
RDA	recommended dietary allowance
RNA	ribonucleic acid
ROS	reactive oxygen species
RSD	relative standard deviation
RT-PCR	reverse transcription-polymerase chain reaction
S9	9000 × *g* supernatant from rat liver
SCE	sister chromatid exchange
SD	standard deviation
SF	serum ferritin
SGPT	serum glutamate–pyruvate transaminase
SGOT	serum glutamate–oxalate transaminase
SPE	solid-phase extraction
T_3	triiodothyronine
T_4	thyroxine
TDI	tolerable daily intake
TfR	serum transferrin receptor
TLC	thin-layer chromatography
TLR	Toll-like receptor
TOS	total organic solids
tRNA	transfer ribonucleic acid
Tsc2	tuberous sclerosis 2
TSH	thyroid stimulating hormone
TTC	threshold of toxicological concern
U	units
UDS	unscheduled deoxyribonucleic acid synthesis
USA	United States of America
USDA	United States Department of Agriculture
USEPA	United States Environmental Protection Agency
UV	ultraviolet
WHO	World Health Organization
w/w	weight per weight

ANNEX 3

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

GENEVA, 19–28 JUNE 2007

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ANNEX 4

ACCEPTABLE DAILY INTAKES, OTHER TOXICOLOGICAL INFORMATION AND INFORMATION ON SPECIFICATIONS

Food additives and ingredients evaluated toxicologically or assessed for dietary exposure

Food additive	Specifications[a]	Acceptable daily intake (ADI) and other toxicological recommendations
Acidified sodium chlorite (ASC)		The available toxicological data were sufficient to assess the safety of ASC by setting ADIs for chlorite and chlorate. Chlorite: ADI of 0–0.03 mg/kg bw Chlorate: ADI of 0–0.01 mg/kg bw New specifications were prepared for sodium chlorite and one of the acids used in the preparation of ASC, sodium hydrogen sulfate.
Asparaginase from *Aspergillus oryzae* expressed in *Aspergillus oryzae*	N	ADI "not specified"[b] when used in the applications specified and in accordance with good manufacturing practice.
Carrageenan and processed *Eucheuma* seaweed	R R	The group ADI "not specified"[b] for the sum of carrageenan and processed *Eucheuma* seaweed was maintained for food additive uses in foods other than infant formula. The Committee was of the view that based on the information available, it is inadvisable to use carrageenan or processed *Eucheuma* seaweed in infant formulas.
Cyclotetraglucose and cyclotetraglucose syrup (listed on draft agenda as cyclotetraose)	N N,T	A temporary ADI "not specified"[b] was allocated for cyclotetraglucose and cyclotetraglucose syrup pending submission of data on the identity of the bacterial strain used to produce the 6-GT/IMT enzyme preparation and evidence of its lack of pathogenicity and toxigenicity. The specifications for cyclotetraglucose syrup were made tentative pending information on the total saccharide content and test methods and the unidentified fraction.
Isoamylase from *Pseudomonas amyloderamosa*	N	ADI "not specified"[b] when used in the applications specified and in accordance with good manufacturing practice.
Magnesium sulfate	R	ADI "not specified"[b].

Food additive	Specifications[a]	Acceptable daily intake (ADI) and other toxicological recommendations
Phospholipase A1 from *Fusarium venenatum* produced by *Aspergillus oryzae*	S	ADI "not specified"[b] when used in the applications specified and in accordance with good manufacturing practice.
Sodium iron(III) ethylenediaminetetraacetic acid (EDTA)	S	Sodium iron EDTA is suitable for use as a source of iron for food fortification to fulfil nutritional iron requirements, provided that the total intake of iron from all food sources including contaminants does not exceed the PMTDI of 0.8 mg/kg bw. Total intake of EDTA should not exceed acceptable levels, also taking into account the intake of EDTA from the food additive use of other EDTA compounds. An ADI of 0–2.5 mg/kg bw was previously established for the calcium disodium and disodium salts of EDTA, equivalent to up to 1.9 mg EDTA/kg bw.
Steviol glycosides	R	The temporary ADI of 0–2 mg/kg bw for steviol glycosides, expressed as steviol, was extended until 2008, pending submission of the results of the ongoing studies. The Committee considered that the newly available data did not raise additional concerns regarding the safety of steviol glycosides, but that the results of ongoing clinical studies, which more closely address the requirements specified at the sixty-third meeting, would be essential to its evaluation. The specifications were revised and the tentative assignation was removed. The method of assay includes a minimum requirement of 95% of the total of seven steviol glycosides.

[a] N: new specifications prepared; R: existing specifications revised; S: existing specifications maintained; T: tentative specifications.

[b] ADI "not specified" is used to refer to a food substance of very low toxicity that, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice, i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.

Food additives, including flavouring agents, considered for specifications only

Food additive		Specifications[a]
Anisyl acetone		W
Furfural		W
Ethyl maltol		R
Maltol		R
Nisin preparation		R
Pectins		R
Polyvinyl alcohol		R
Sucrose esters of fatty acids		R
Zeaxanthin-rich extract from *Tagetes erecta*		W
Flavouring agent	**JECFA No.**	**Specifications[a]**
3-Acetyl-2,5-dimethylfuran	1506	R
Ethyl maltol	1481	R
Maltol	1480	R
Maltyl isobutyrate	1482	R
3-Methyl-2-oxobutanoic acid	631	R
3-Methyl-2-oxopentanoic acid	632	R
4-Methyl-2-oxopentanoic acid	633	R
Sodium 3-methyl-2-oxobutanoate	631.1	R
Sodium 3-methyl-2-oxopentanoate	632.1	R
Sodium 4-methyl-2-oxopentanoate	633.1	R
Sodium 2-oxo-3-phenylpropionate	1479	R
2,4,5-Trimethyl-delta-oxazolin	1559	R

[a] R: existing specifications revised; W: existing specifications withdrawn.

Food contaminants evaluated toxicologically or assessed for dietary exposure

Food contaminant	Tolerable intakes and other toxicological recommendations
Aflatoxins (AFL) (Intake assessment from almonds, Brazil nuts, hazelnuts, pistachios and dried figs, impact of various MLs)	The Committee decided to base the assessment of the impact of different MLs for AFL exposure on data provided by producing countries, noting that these better represent the materials in commerce and result in a robust estimate of dietary AFL exposure from the tree nuts. Consumption of almonds, Brazil nuts, hazelnuts, pistachios and dried figs contributes greater than 5% of the total dietary AFL exposure in only 5 of the 13 GEMS/Food Consumption Cluster Diets (clusters B, C, D, E and M). If fully enforced, an ML at 20 µg/kg in almonds, Brazil nuts, hazelnuts, pistachios and dried figs would have an impact on the relative contribution to dietary AFL exposure only in these clusters, including high-level consumers of tree nuts. This contribution is due solely to the elevated AFL level in pistachios. For tree nuts other than pistachios, the presence of an ML has no effect on dietary AFL exposure. Moreover, the Committee concluded that enforcing an ML of 15, 10, 8 or 4 µg/kg

Food contaminant	Tolerable intakes and other toxicological recommendations
	would have little further impact on the overall dietary exposure to AFL in all five of the highest exposed population groups compared with setting an ML of 20 µg/kg.
	Regarding dried figs, the Committee concluded that whatever the hypothetical ML scenario applied (no ML, 4, 8, 10, 15 or 20 µg/kg), there would be no impact on the overall dietary exposure to AFL.
	The Committee noted that the reduction of dietary AFL exposure is an important public health goal, particularly in populations that consume high levels of any potentially AFL-contaminated food.
Ochratoxin A	The previous PTWI of 100 ng/kg bw was retained.
	The new data, including data on mode of action of ochratoxin A in the kidney, do not indicate any reason to modify the previous risk assessment approach taken by JECFA.
	The current estimate of overall dietary exposure to ochratoxin A from cereals, based mainly on European data, is about 8–17 ng/kg bw per week, based on processed cereals, compared with 25 ng/kg bw per week in the previous evaluation, based on raw cereals. The current estimates are well below the PTWI.
	Contamination levels in the majority of raw cereal samples were below 5 µg/kg. Owing to the very small number of samples contaminated above the highest proposed limit of 20 µg/kg, such an ML would have very limited impact compared with no ML. The Committee concluded that the use of an ML of 5 or 20 µg/kg would be unlikely to have an impact on dietary exposure to ochratoxin A. The Committee was unable to reach a conclusion regarding the situation in developing countries, owing to the lack of adequate data to consider.

Flavouring agents evaluated using the Procedure for the Safety Evaluation of Flavouring Agents

A. Linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Ethyl-2-methyl-3,4-pentadienoate	353	S	No safety concern
Methyl 4-pentenoate	1616	N	No safety concern
2-Methylbut-2-en-1-ol	1617	N	No safety concern
Ethyl 4-pentenoate	1618	N	No safety concern
4-Pentenal	1619	N	No safety concern
3-Isopropenylpentanedioic acid	1620	N	No safety concern
trans-3-Hexenol	1621	N	No safety concern
trans-4-Hexenal	1622	N	No safety concern
5-Hexenol	1623	N	No safety concern

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Methyl (*Z*)-3-hexenoate	1624	N	No safety concern
cis-4-Octenol	1625	N	No safety concern
Ethyl (*Z*)-3-hexenoate	1626	N	No safety concern
3-Octenoic acid	1627	N	No safety concern
(*Z*)-3-Octenyl propionate	1628	N	No safety concern
trans-4-Octenoic acid	1629	N	No safety concern
Methyl (*Z*)-5-octenoate	1630	N	No safety concern
cis-5-Octenoic acid	1631	N	No safety concern
Ethyl 3-octenoate	1632	N	No safety concern
cis-4-Decenol	1633	N	No safety concern
Isobutyl 10-undecenoate	1634	N	No safety concern
11-Dodecenoic acid	1635	N	No safety concern
(*Z*)-4-Dodecenal	1636	N	No safety concern
cis-9-Octadecenol	1637	N	No safety concern
cis-9-Octadecenyl acetate	1638	N	No safety concern
Methyl 10-undecenoate	1639	N	No safety concern
(*Z*)-8-Tetradecenal	1640	N	No safety concern
9-Octadecenal	1641	N	No safety concern
(*E*)-4-Nonenal	1642	N	No safety concern

[a] N: new specifications prepared; S: existing specifications maintained.

B. Aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class I			
2,3,4-Trimethyl-3-pentanol	1643	N	No safety concern
(±)-2,4,8-Trimethyl-7-nonen-2-ol	1644	N	No safety concern
(*E*)- and (*Z*)-2,4,8-Trimethyl-3,7-nonadien-2-ol	1645	N	No safety concern
Nerolidol	1646	N	No safety concern
1-Phenyl-3-methyl-3-pentanol	1649	N	No safety concern
p-α,α-Trimethylbenzyl alcohol	1650	N	No safety concern
(±)-Ethyl 2-hydroxy-2-methylbutyrate	1651	N	No safety concern
(±)-Ethyl 2-hydroxy-3-methylvalerate	1652	N	No safety concern
α,α-Dimethylphenethyl alcohol	1653	N	No safety concern
α,α-Dimethylphenethyl formate	1654	N	No safety concern
α,α-Dimethylphenethyl acetate	1655	N	No safety concern
α,α-Dimethylphenethyl butyrate	1656	N	No safety concern
α,α-Dimethylbenzyl isobutyrate	1657	N	No safety concern

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class II			
6-Acetoxydihydrotheaspirane	1647	N	No safety concern
6-Hydroxydihydrotheaspirane	1648	N	No safety concern

[a] N: new specifications prepared.

C. Simple aliphatic and aromatic sulfides and thiols

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Simple sulfides			
Structural class I			
2-Methyl-1-methylthio-2-butene	1683	N	No safety concern
2,4,6-Trithiaheptane	1684	N	No safety concern
2,5-Dithiahexane	1707	N	No safety concern
Acyclic sulfides with oxidized and thiol side-chains			
Structural class I			
Methionyl butyrate	1668	N	No safety concern
Methylthiomethylmercaptan	1675	N	No safety concern
(±)-Isobutyl 3-methylthiobutyrate	1677	N	No safety concern
3-(Methylthio)-2-butanone	1688	N	No safety concern
4-(Methylthio)-2-pentanone	1689	N	No safety concern
Methyl 3-(methylthio)butanoate	1690	N	No safety concern
Methyl (methylthio)acetate	1691	N	No safety concern
(±)-3-(Methylthio)heptanal	1692	N	No safety concern
(±)-3-(Ethylthio)butanol	1703	N	No safety concern
S-Allyl-L-cysteine	1710	N	No safety concern
Heterocyclic sulfides			
Structural class I			
(±)-2,8-Epithio-*cis*-*p*-menthane	1685	N	No safety concern
Simple thiols			
Structural class I			
Ethanethiol	1659	N	No safety concern
1-Pentanethiol	1662	N	No safety concern
Heptane-1-thiol	1663	N	No safety concern
2-Heptanethiol	1664	N	No safety concern
Structural class II			
(±)-1-Phenylethylmercaptan	1665	N	No safety concern
Thiols with oxidized side-chains			
Structural class I			
Propyl 2-mercaptopropionate	1667	N	No safety concern
(±)-4-Mercapto-4-methyl-2-pentanol	1669	N	No safety concern

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
4-Mercapto-2-pentanone	1670	N	No safety concern
(*S*)-1-Methoxy-3-heptanethiol	1671	N	No safety concern
Methyl 3-mercaptobutanoate	1674	N	No safety concern
Hexyl 3-mercaptobutanoate	1704	N	No safety concern
(±)-3-Mercapto-1-butyl acetate	1705	N	No safety concern
3-Mercapto-3-methyl-1-butyl acetate	1706	N	No safety concern
3-Mercaptoheptyl acetate	1708	N	No safety concern
Structural class II			
cis- and *trans*-Mercapto-*p*-menthan-3-one	1673	N	No safety concern
Structural class III			
2-Mercaptoanisole	1666	N	No safety concern
Diisopentyl thiomalate	1672	N	No safety concern
Dithiols			
Structural class I			
Ethane-1,1-dithiol	1660	N	No safety concern
Dimercaptomethane	1661	N	No safety concern
bis(1-Mercaptopropyl)sulfide	1709	N	No safety concern
Simple disulfides			
Structural class I			
Ethyl methyl disulfide	1693	N	No safety concern
Ethyl propyl disulfide	1694	N	No safety concern
Methyl isopentyl disulfide	1696	N	No safety concern
Amyl methyl disulfide	1697	N	No safety concern
Butyl ethyl disulfide	1698	N	No safety concern
Diethyl disulfide	1699	N	No safety concern
Structural class II			
Allyl propyl disulfide	1700	N	No safety concern
Trisulfides			
Structural class I			
Ethyl propyl trisulfide	1695	N	No safety concern
Diethyl trisulfide	1701	N	No safety concern
Heterocyclic disulfides			
Structural class II			
(±)-3,5-Diethyl-1,2,4-trithiolane	1686	N	No safety concern
Mixture of 3,6-diethyl-1,2,4,5-tetrathiane (approx. 55%) and 3,5-diethyl-1,2,4-trithiolane (approx. 45%)	1687	N	No safety concern
Thioesters and acids			
Structural class I			
Thioacetic acid	1676	N	No safety concern
(*S*)-Methyl propanethioate	1678	N	No safety concern
(*S*)-Isopropyl 3-methylbut-2-enethioate	1679	N	No safety concern
Structural class II			
Allyl thiohexanoate	1681	N	No safety concern
Structural class III			

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
(*S*)-Ethyl 2-acetylaminoethanethioate	1680	N	No safety concern
Propyl propane thiosulfonate	1702	N	No safety concern

[a] N: new specifications prepared.

D. Aliphatic acyclic diols, triols and related substances

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class I			
Dihydroxyacetone dimer	1716	N	No safety concern
1-Hydroxy-2-butanone	1717	N	No safety concern
Ethyl 3-acetoxy-2-methylbutyrate	1718	N	No safety concern
Methyl 5-acetoxyhexanoate	1719	N	No safety concern
Structural class III			
2,4-Dimethyl-1,3-dioxolane	1711	N	No safety concern
2-Hexyl-4,5-dimethyl-1,3-dioxolane	1712	N	No safety concern
cis- and *trans*-Ethyl 2,4-dimethyl-1,3-dioxolane-2-acetate	1715	N	No safety concern

[a] N: new specifications prepared.

Five substances in this group (listed as Nos 1720, 1721 and 1723–1725 in the Call for data; they are various fatty acid esters of glycerol and propylene glycol) had been previously evaluated by the Committee as emulsifying agents. These substances have food additive specifications and have been allocated ADIs. Although the use of these substances as flavouring agents would not be anticipated to cause a safety concern, the Committee questioned whether these substances have flavouring properties and did not evaluate them according to the Procedure for the Safety Evaluation of Flavouring Agents. In addition, the Committee questioned the flavouring function of lactylated fatty acid esters of glycerol and propylene glycol (listed as No. 1722), for which an ADI and specifications are not available, and decided not to evaluate this substance as a flavouring agent using the Procedure.

E. Aliphatic acetals

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class I			
(±)-1-Acetoxy-1-ethoxyethane	1726	N	No safety concern

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Acetaldehyde hexyl isoamyl acetal	1727	N	No safety concern
1,1-Dimethoxy-*trans*-2-hexene	1728	N	No safety concern
Acetaldehyde diisoamyl acetal	1729	N	No safety concern
Isovaleraldehyde diethyl acetal	1730	N	No safety concern
Valeraldehyde dibutyl acetal	1731	N	No safety concern
Hexanal hexyl isoamyl acetal	1735	N	No safety concern
Hexanal dihexyl acetal	1738	N	No safety concern
Nonanal dimethyl acetal	1742	N	No safety concern
Dodecanal dimethyl acetal	1746	N	No safety concern
Acetaldehyde di-*cis*-3-hexenyl acetal	1747	N	No safety concern
Structural class III			
Isovaleraldehyde propyleneglycol acetal	1732	N	No safety concern
Isovaleraldehyde glyceryl acetal	1733	N	No safety concern
Valeraldehyde propyleneglycol acetal	1734	N	No safety concern
Hexanal octane-1,3-diol acetal	1736	N	No safety concern
Hexanal butane-2,3-diol acetal	1737	N	No safety concern
Heptanal propyleneglycol acetal	1739	N	No safety concern
2,6-Dimethyl-5-heptenal propyleneglycol acetal	1740	N	No safety concern
Octanal propyleneglycol acetal	1741	N	No safety concern
Nonanal propyleneglycol acetal	1743	N	No safety concern
Decanal propyleneglycol acetal	1744	N	No safety concern
Undecanal propyleneglycol acetal	1745	N	No safety concern
Isobutanal propyleneglycol acetal	1748	N	No safety concern
Acetaldehyde 1,3-octanediol acetal	1749	N	No safety concern

[a] N: new specifications prepared.

F. Sulfur-containing heterocyclic compounds

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class II			
1-(3-Hydroxy-5-methyl-2-thienyl)ethanone	1750	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl formate	1751	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl propionate	1752	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl butanoate	1753	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl isobutyrate	1754	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl hexanoate	1755	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl octanoate	1756	N	No safety concern
2-(4-Methyl-5-thiazolyl)ethyl decanoate	1757	N	No safety concern
2,5-Dimethylthiazole	1758	N	No safety concern
2-Acetyl-2-thiazoline	1759	N	No safety concern

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
2-Propionyl-2-thiazoline	1760	N	No safety concern
2-Hexylthiophene	1764	N	No safety concern
5-Acetyl-2,3-dihydro-1,4-thiazine	1766	N	No safety concern
Structural class III			
cis- and *trans*-5-Ethyl-4-methyl-2-(2-methylpropyl)thiazoline	1761	N	No safety concern
cis- and *trans*-5-Ethyl-4-methyl-2-(1-methylpropyl)thiazoline	1762	N	No safety concern
Pyrrolidino-[1,2e]-4H-2,4-dimethyl-1,3,5-dithiazine	1763	N	No safety concern
3-(Methylthio)methylthiophene	1765	N	No safety concern

[a] N: new specifications prepared.

G. Aliphatic and aromatic amines and amides

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class I			
4-Aminobutyric acid	1771	N	No safety concern
N-Gluconyl ethanolamine	1772	N	No safety concern
N-Gluconyl ethanolamine phosphate	1773	N	No safety concern
N-Lactoyl ethanolamine	1774	N	No safety concern
N-Lactoyl ethanolamine phosphate	1775	N	No safety concern
Structural class III			
N-(Heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide	1767	N	No safety concern
*N*1-(2,4-Dimethoxybenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	1768	N	No safety concern
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(5-methylpyridin-2-yl)ethyl)oxalamide	1769	N	No safety concern
*N*1-(2-Methoxy-4-methylbenzyl)-*N*2-(2-(pyridin-2-yl)ethyl)oxalamide	1770	N	No safety concern
N-[(Ethoxycarbonyl)methyl]-*p*-menthane-3-carboxamide	1776	N	No safety concern
N-[2-(3,4-Dimethoxyphenyl)ethyl]-3,4-dimethoxycinnamic acid amide	1777	N	No safety concern
N-3,7-Dimethyl-2,6-octadienyl cyclopropylcarboxamide	1779	N	No safety concern

[a] N: new specifications prepared.

H. Aliphatic alicyclic linear α,β-unsaturated di- and trienals and related alcohols, acids and esters

Flavouring agent	JECFA No.	Specifications[a]	Conclusions based on current estimated intake
Structural class I			
2,4-Hexadienyl acetate	1780	N	No safety concern
2,4-Hexadienyl propionate	1781	N	No safety concern
2,4-Hexadienyl isobutyrate	1782	N	No safety concern
2,4-Hexadienyl butyrate	1783	N	No safety concern
2,4-Heptadien-1-ol	1784	N	No safety concern
Nona-2,4,6-trienal	1785	N	No safety concern
2,4,7-Decatrienal	1786	N	No safety concern

[a] N: new specifications prepared.

ANNEX 5

SUMMARY OF THE SAFETY EVALUATION OF SECONDARY COMPONENTS FOR FLAVOURING AGENTS WITH MINIMUM ASSAY VALUES OF LESS THAN 95%

JECFA No.	Flavouring agent	Minimum assay value (%)	Secondary components	Comments on secondary components
Linear and branched-chain aliphatic, unsaturated, unconjugated alcohols, aldehydes, acids and related esters				
1622	*trans*-4-Hexenal	76	16–20% *cis*-4-hexenal, 2–4% *cis*-3-hexen-1-ol, 1–2% hexanal	*cis*-4-Hexenal (No. 319) was evaluated by the Committee in 1998. It was concluded that *cis*-4-hexenal was not a safety concern at current levels of intake. *cis*-3-Hexen-1-ol (No. 315) was evaluated by the Committee in 1998. It was concluded that *cis*-3-hexen-1-ol was not a safety concern at current levels of intake. In a 98-day rat drinking-water study, *cis*-3-hexen-1-ol exhibited a NOEL of 120–180 mg/kg bw per day (Gaunt et al., 1969). Hexanal (No. 92) was evaluated by the Committee in 1997. It was concluded that hexanal was not a safety concern at current levels of intake.
1636	(*Z*)-4-Dodecenal	94	3–4% dodecanal	Dodecanal is expected to share the same metabolic fate as (*Z*)-4-dodecenal and the other saturated and unsaturated aliphatic alcohols, aldehydes, carboxylic acids and related esters in this group (Dawson et al., 1964; Gaillard & Derache, 1965).
1637	*cis*-9-Octadecenol	85	8–9% hexadecanol, 5–6% octadecanol	Hexadecanol and octadecanol are expected to share the same metabolic fate as *cis*-9-octadecenol and the other saturated and unsaturated aliphatic alcohols, aldehydes, carboxylic acids and related esters in this group (Dawson et al., 1964; Gaillard & Derache, 1965).

1638	*cis*-9-Octadecenyl acetate	92	2–3% hexadecyl acetate, 2–3% octadecyl acetate	Hexadecyl acetate and octadecyl acetate are anticipated to share the same metabolic fate as *cis*-9-octadecenyl acetate and the other alcohols, aldehydes, carboxylic acids and related esters in this group (Gangolli & Shilling, 1968; Longland et al., 1977; Drake et al., 1978; Heymann, 1980; Graffner-Nordberg et al., 1998; Hosokawa et al., 2001).
1641	9-Octadecenal	94	3–5% octadecenal	Octadecenal is anticipated to share the same metabolic fate as 9-octadecenal and the other alcohols, aldehydes, carboxylic acids and related esters in this group (Dawson et al., 1964; Gaillard & Derache, 1965).
1642	(*E*)-4-Nonenal	93	1–2% 2-nonen-4-ol, 5–6% 2*E*,4*E*-nonadienal	2-Nonen-4-ol and 2*E*,4*E*-nonadienal are anticipated to share the same metabolic fate as (*E*)-4-nonenal and the other alcohols, aldehydes, carboxylic acids and related esters in this group (Dawson et al., 1964; Gaillard & Derache, 1965).
Aliphatic acyclic and alicyclic terpenoid tertiary alcohols and structurally related substances				
1650	*p*-α,α-trimethylbenzyl alcohol	90	9–11% *p*-isopropenyltoluene	*p*-Isopropenyltoluene (No. 1333, *p*,α-dimethylstyrene) was evaluated by the Committee in 2004. It was concluded that *p*,α-dimethylstyrene was not a safety concern at current levels of intake. In a 90-day rat study, *p*,α-dimethylstyrene exhibited NOELs of 0.63 and 0.62 mg/kg bw per day for males and females, respectively (Posternak et al., 1969).
1654	α,α-Dimethylphenethyl formate	93	5–7% α,α-dimethylphenethyl alcohol	α,α-Dimethylphenethyl alcohol is anticipated to share the same metabolic fate as the other tertiary terpenoid alcohols in this group (Williams, 1959; Parke et al., 1974; Horning et al., 1976; Ventura et al., 1985).

Simple aliphatic and aromatic sulfides and thiols

1660	Ethane-1,1-dithiol	1	Owing to malodorous nature, available only as a 1% solution in ethanol	Ethanol (No. 41) was evaluated by the Committee in 1996. It was concluded that ethanol was not a safety concern at current levels of intake.
1670	4-Mercapto-2-pentanone	1	Owing to malodorous nature, available only as a 1% solution in acetoin	Acetoin (No. 405) was evaluated by the Committee in 1998. It was concluded that acetoin was not a safety concern at current levels of intake.
1672	Diisopentyl thiomalate	94	2–3% diisopentyl thiotartronate	Diisopentyl thiotartronate is anticipated to undergo simultaneous metabolism of sulfur and oxygenated functional groups (Gachon et al., 1988; Karim et al., 1988; Feng & Solsten, 1991; Wilson et al., 1991; Black et al., 1993). Sulfoxide formation is usually the predominant metabolic detoxication pathway.
1673	*cis*- and *trans*-Mercapto-*p*-menthan-3-one	89	8–9% piperitone, 1–2% α-terpineol	Piperitone (No. 435) was evaluated by the Committee in 1998. It was concluded that piperitone was not a safety concern at current levels of intake. α-Terpineol (No. 366) was evaluated by the Committee in 1998. It was concluded that α-terpineol was not a safety concern at current levels of intake.
1684	2,4,6-Trithiaheptane	10	Owing to malodorous nature, available only as a 10% solution in triacetin	Triacetin (No. 920) was evaluated by the Committee in 2002. It was concluded that triacetin was not a safety concern at current levels of intake.
1685	(±)-2,8-Epithio-*cis*-*p*-menthane	93	5–6% d-limonene	d-Limonene (No. 1324) was evaluated by the Committee in 2004. It was concluded that d-limonene was not a safety concern at current levels of intake.
1687	Mixture of 3,6-diethyl-1,2,4,5-tetrathiane and 3,5-diethyl-1,2,4-trithiolane	1	Owing to malodorous nature, available only as a 1% solution in vegetable oil	Vegetable oil is a common component of traditional foods.

1692	(±)-3-(Methylthio)heptanal	92	5–7% 2-(*E*)-heptenal	2-(*E*)-Heptenal (No. 1360, *trans*-2-heptenal) was evaluated by the Committee in 2004. It was concluded that 2-(*E*)-heptenal was not a safety concern at current levels of intake.
1693	Ethyl methyl disulfide	80	8–10% dimethyl disulfide, 7–8% diethyl disulfide	Dimethyl disulfide (No. 564) was evaluated by the Committee in 1999. It was concluded that dimethyl disulfide was not a safety concern at current levels of intake. Diethyl disulfide (No. 1699) was evaluated by the Committee at the present meeting. It was concluded that diethyl disulfide was not a safety concern at current levels of intake. Diethyl disulfide is anticipated to undergo reduction to ethylthiol with subsequent methylation. Ethyl methyl sulfide is oxidized and eliminated in the urine (Snow, 1957).
1695	Ethyl propyl trisulfide	50	20–30% diethyl trisulfide, 20–30% dipropyl trisulfide	Diethyl trisulfide (No. 1701) was evaluated by the Committee at the present meeting. It was concluded that diethyl trisulfide was not a safety concern at current levels of intake. Diethyl trisulfide is predicted to be converted rapidly to the corresponding disulfide with subsequent reduction to thiol (Moutiez et al., 1994), which is then metabolized via the various pathways for simple thiols. Dipropyl trisulfide (No. 585) was evaluated by the Committee in 1999. It was concluded that dipropyl trisulfide was not a safety concern at current levels of intake.

1696	Methyl isopentyl disulfide	92	3–5% crotonic acid	Crotonic acid (No. 1371, (*E*)-2-butenoic acid) was evaluated by the Committee in 2004. It was concluded that (*E*)-2-butenoic acid was not a safety concern at current levels of intake.
1698	Butyl ethyl disulfide	90	2–3% diethyl disulfide, 5–6% dibutyl disulfide	Diethyl disulfide (No. 1699) was evaluated by the Committee at the present meeting. It was concluded that diethyl disulfide was not a safety concern at current levels of intake. Diethyl disulfide is anticipated to undergo reduction to ethylthiol with subsequent methylation. Ethyl methyl sulfide is oxidized and eliminated in the urine (Snow, 1957). Dibutyl disulfide is anticipated to undergo reduction to the corresponding thiol, which will be methylated. Butyl methyl sulfide will be oxidized and eliminated in the urine (Snow, 1957).
1700	Allyl propyl disulfide	93	1–2% allyl propyl sulfide, 1–2% dipropyl sulfide	Allyl propyl sulfide and dipropyl sulfide are anticipated to undergo rapid oxidation to form the corresponding sulfoxides and potentially sulfones, which are eliminated in the urine (Damani, 1987).
1709	bis-(1-Mercaptopropyl)sulfide	56	36% 3,5-diethyl-1,2,4-trithiolane, approximately 5% dipropyl trisulfide	3,5-Diethyl-1,2,4-trithiolane (No. 1686) was evaluated by the Committee at the present meeting. It was concluded that 3,5-diethyl-1,2,4-trithiolane was not a safety concern at current levels of intake. 3,5-Diethyl-1,2,4-trithiolane is anticipated to undergo oxidation and subsequent elimination in the urine or reduction to the free dithiol (Nelson & Cox, 2000). Dipropyl trisulfide (No. 585) was evaluated by the Committee in 1999. It was concluded that dipropyl trisulfide was not a safety concern at current levels of intake.

1717	1-Hydroxy-2-butanone	90	5–10% acetoin	Acetoin (No. 405) was evaluated by the Committee in 1998. It was concluded that acetoin was not a safety concern at current levels of intake.
Aliphatic and aromatic amines and amides				
1774	*N*-Lactoyl ethanolamine	90	6–8% 2-aminoethanol lactate	2-Aminoethanol lactate is anticipated to undergo hydrolysis to form ethanolamine and lactic acid (Schmid et al., 1985). Lactic acid (No. 930) was evaluated by the Committee in 2001. It was concluded that lactic acid was of no safety concern at current levels of intake. 2-Aminoethanol is anticipated to undergo conjugation with glucuronic acid via the alcohol moiety, or, as a primary aliphatic amine with an accessible α-substituted carbon atom, it may be *N*-oxidized to nitroso groups and subsequently oximes by cytochrome P450 enzymes (Uehleke, 1973).
1775	N-Lactoyl ethanolamine phosphate	90	6–10% ammonium formate	Ammonium formate is anticipated to hydrolyse to form ammonia and formic acid. Formic acid (No. 79) was evaluated by the Committee in 1997. It was concluded that formic acid was not a safety concern at current levels of intake. Ammonia found in the intestinal lumen by either ingestion or endogenous production is rapidly absorbed into the portal vein and converted to urea by the liver via the Krebs-Henseleit urea cycle (Furst et al., 1969; Pitts, 1971; Mathews & van Holde, 1990; Nelson & Cox, 2000).

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The toxicological monographs in this volume summarize the safety data on a number of food additives: acidified sodium chlorite, asparaginase from Aspergillus oryzae expressed in Aspergillus oryzae, carrageenan and processed Euchema seaweed, cyclotetraglucose and cyclotetraglucose syrup, isoamylase from Pseudomonas amyloderamosa, magnesium sulfate, phospholipase A1 from Fusarium venenatum expressed in Aspergillus oryzae, sodium iron(III) ethylenediaminetetraacetic acid (EDTA) and steviol glycosides.

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Seventy-ninth meeting of the Joint FAO/WHO Expert Committee on Food Additives

Geneva, 17–26 June 2014

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List of abbreviations

ADI	acceptable daily intake
bw	body weight
CAS	Chemical Abstracts Service
CCCF	Codex Committee on Contaminants in Foods
CCFA	Codex Committee on Food Additives
CITREM	citric and fatty acid esters of glycerol
DNA	deoxyribonucleic acid
EFSA	European Food Safety Authority
Eq	equivalents
EU-16	Austria, Belgium, Cyprus, Finland, France, Germany, Greece, Ireland, Italy, Latvia, Luxembourg, Malta, Portugal, Slovakia, Slovenia and Spain
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association of the United States
GLP	good laboratory practice
GRAS	Generally Recognized as Safe
GSFA	(Codex) General Standard for Food Additives
HLB	hydrophilic–lipophilic balance
IL	interleukin
INS	International Numbering System for Food Additives
IOFI	International Organization of the Flavor Industry
JECFA	Joint FAO/WHO Expert Committee on Food Additives
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
ML	maximum level
MOE	margin of exposure
MSDI	maximized survey-derived intake
NMR	nuclear magnetic resonance
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
OSA	octenyl succinic acid
pAOS	pectin-derived acidic oligosaccharides
PAS	potassium aluminium silicate
SPET	single-portion exposure technique
TNF	tumour necrosis factor
TTC	threshold of toxicological concern
USA	United States of America
WHO	World Health Organization
w/w	weight per weight

Dedication

Dr John Christian Larsen

National Food Institute, Technical University of Denmark (retired)

It was with great sadness that the Committee noted the passing of Dr John Christian Larsen. John Christian served on the Committee since the early 1990s in various capacities, including as Rapporteur and as Chair. He chaired many meetings, and in his unique, quiet and thoughtful way, he led the Committee to successful outcomes. With his leadership and breadth of scientific knowledge, he guided the Committee through discussions that at times were difficult and controversial. He brought not only dedication and thoroughness to the work of the Committee, but also a human touch. John Christian will be thoroughly missed by his peers and friends in the scientific community.

In recognition of his services, the Committee dedicated this report to the memory of Dr John Christian Larsen.

Monographs containing summaries of relevant data and toxicological evaluations are available from WHO under the title:

Safety evaluation of certain food additives. WHO Food Additives Series, No. 70, 2014.

Specifications are issued separately by FAO under the title:

Compendium of food additive specifications. FAO JECFA Monographs 16, 2014.

1. Introduction

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) met in Geneva from 17 to 26 June 2014. The meeting was opened by Dr Kazuaki Miyagishima, Director of the Department of Food Safety and Zoonoses of the World Health Organization (WHO), who welcomed participants on behalf of the Directors-General of the Food and Agriculture Organization of the United Nations (FAO) and WHO. Dr Miyagishima commented that JECFA was one of the most successful joint undertakings of FAO and WHO, playing a critical role in the development of international food safety standards by the Codex Alimentarius Commission. He noted that although the integrity and quality of the work of JECFA are sometimes questioned by the media or activist groups, WHO and FAO will defend the Committee or individual participants who are unjustly attacked.

Dr Miyagishima mentioned several ongoing initiatives and discussions of relevance to food safety. The Millennium Development Goals will be coming to the end of their term in 2015, and the United Nations is preparing to set a series of new targets to support sustainable development worldwide. The work of JECFA plays an important role in the provision of safe, affordable and nutritious food – one of the focus areas in the discussion on the sustainable development goals of the United Nations – by providing the scientific basis for policy decisions. An international conference on nutrition (ICN2), to be held in Rome in November 2014, will include some discussion on food safety, and Milan will host the World Expo 2015 with a focus on food, which will be another opportunity to highlight international work on food safety.

One of the tasks before this Committee is to evaluate the safety of four food additives for specific use in infant formulas, as requested by the Codex Committee on Food Additives (CCFA). Another task is the evaluation of several plant extracts for use as colours, which presents a unique challenge in terms of ensuring that the products in commerce are clearly defined and safe for their intended uses. One of the important general considerations on the agenda is related to the application of the principle of the threshold of toxicological concern (TTC) for the evaluation of flavouring agents. Another important general consideration relates to the limit of lead in the specifications for food additives intended for use in infant foods.

Dr Miyagishima reminded participants that they have been invited to this meeting as independent experts and not as representatives of their countries or organizations. He also reminded them of the confidential nature of this meeting, which allows experts to freely express their opinions. He closed by expressing his sincere gratitude to participants for providing their time and expertise to this important work, which contributes to the core business of both FAO and WHO, providing science-based international norms and standards.

1.1 Declarations of interests

The Secretariat informed the Committee that all experts participating in the seventy-ninth meeting had completed declaration of interest forms and that no conflicts of interest were identified. The following declared interests and potential conflicts were discussed by the Committee. Data on gardenia yellow and polyoxyethylene (20) sorbitan monostearate were submitted from the institution where Dr Yoko Kawamura is working, although she was not involved in the preparation of the data submission and was not assigned to the evaluation of these substances. Professor Gary Williams' research group received a research grant through his university from the International Organization of the Flavor Industry (IOFI) to investigate the toxicological mode of action for a flavour compound not on the agenda; and he served on the Generally Recognized as Safe (GRAS) panel for Intertek for the evaluation of polysorbates (on the agenda of this meeting for revision of specifications only). Professor Glenn Sipes participated on several GRAS panels, none of them on any compounds on the agenda. He also reviewed a document on octenyl succinic acid (OSA)–modified starch for Intertek. He was not aware if this work was related to the submission, but he did not participate in the discussion on this compound. Dr Josef Schlatter served on an expert panel of the Flavor and Extract Manufacturers Association of the United States (FEMA) that discussed three flavouring compounds on the agenda of this meeting. He did not participate in discussions on these flavouring agents at this meeting.

1.2 Modification of the agenda

The Committee made the following modifications to the agenda (see original agenda in Annex 4):

- Following the request from CCFA to consider the deletion of the functional use of carrier from the specifications for potassium aluminium silicate (PAS) (INS 555), the Committee agreed to add this item under agenda item 7.2 (food additives for revision of specifications only).
- *Tagetes* extract was on the agenda at the request of CCFA to undertake a safety assessment and revision of specifications. The Committee noted that the information supplied by the sponsor referred to a substance of a higher carotenoid ester content; therefore, it was decided to name it "Lutein esters from *Tagetes erecta*" to differentiate it from *Tagetes* extract.
- The Committee determined that the flavouring agent α-ionene (No. 2193) did not belong in the flavouring agent group of aliphatic and alicyclic hydrocarbons and did not evaluate it at the meeting.

- The Committee was able to perform a safety evaluation of Benzoe tonkinensis, so it was moved from agenda item 7.2 (food additives for revision of specifications only) to agenda item 7.1 (toxicological evaluations).

2. General considerations

As a result of the recommendations of the first Joint FAO/WHO Conference on Food Additives, held in September 1955 *(1)*, there have been 78 previous meetings of the Committee (Annex 1). The present meeting was convened on the basis of a recommendation made at the seventy-seventh meeting (Annex 1, reference *214*).

The tasks before the Committee were to:

- elaborate further principles for evaluating the safety of food additives (including flavouring agents) (section 2);
- review and prepare specifications for certain food additives (including flavouring agents) (section 3 and Annex 2);
- undertake safety evaluations of certain food additives (including flavouring agents) (sections 3 and 4 and Annex 2).

2.1 Report from the Forty-sixth Session of the Codex Committee on Food Additives (CCFA)

The Codex Secretariat provided the Committee with an update on the work of CCFA since the seventy-seventh meeting of JECFA (Annex 1, reference *214*).

Following the seventy-seventh meeting of JECFA, the Forty-sixth Session of CCFA *(2)* agreed to wait for further evaluation of advantame (INS 969) and OSA-modified gum arabic (INS 423) and removed Note 28 on nisin acceptable daily intake (ADI) conversion from the Codex General Standard for Food Additives (GSFA) *(3)*. It also recommended the inclusion of glucoamylase from *Trichoderma reesei* expressed in *Trichoderma reesei* in the database on processing aids prepared by China (www.ccfa.cc/IPA) and noted that no action was required for glycerol ester of tall oil rosin (INS 445(i)) or glycerol ester of wood rosin (INS 445(ii)).

Work on more than 700 provisions of the GSFA was finalized, and the adoption of new and revised specifications for the identity and purity of 10 food additives, prepared by the seventy-seventh meeting of JECFA, was recommended. With regard to PAS (INS 555), CCFA requested JECFA to consider the deletion of the functional use of carrier from the specifications.

The Forty-sixth Session of CCFA agreed on a revised priority list of substances for evaluation (or re-evaluation) by JECFA and assigned high priority to eight of them. CCFA also included benzoates on the priority list for exposure assessment. With regard to the food additives included in the GSFA for which there were no corresponding JECFA specifications, CCFA removed from the GSFA the food additives for which no information on their commercial use was provided and included the others on the priority list subject to confirmation of

the provision of dossiers by its next session. With regard to the re-evaluation of substances, CCFA agreed to a process on a trial basis, which would use six prioritized food colours as a working example. These colours will be added to the priority list, under a separate table, subject to confirmation of the availability of data to the next session of CCFA.

The Forty-sixth Session of CCFA completed work on the alignment of the food additive provisions in five standards for meat products and the corresponding provisions of the GSFA and agreed to continue working on the food additive provisions of other standards. Work on alignment will result in the GSFA becoming the only reference for food additives in Codex. Only additives evaluated by JECFA can be included in the GSFA.

The Forty-sixth Session of CCFA completed work on the Guidelines for the Simple Evaluation of Dietary Exposure to Food Additives *(4)*. The Guidelines are intended to facilitate the work of countries with limited resources on the assessment of dietary exposure to food additives. CCFA further agreed to consider at its next session a document on secondary additives, which will address a definition and analyse the issues and potential inconsistencies in the CCFA work.

2.2 Principles governing the toxicological evaluation of compounds on the agenda

In making recommendations on the safety of food additives, the Committee took into consideration the principles established and contained in the publication, Environmental Health Criteria, No. 240, *Principles and Methods for the Risk Assessment of Chemicals in Food*, published in 2009 *(5)*.

2.3 Threshold of toxicological concern (TTC) principle: update on a WHO project and implications for the Procedure for the Safety Evaluation of Flavouring Agents

The Committee was informed about a project that WHO is undertaking in collaboration with the European Food Safety Authority (EFSA) on a review of the application of the TTC principle in the risk assessment of chemicals, based on the current state-of-the-science and building on existing work.

A draft report was presented reviewing the Cramer classification scheme *(6)*, with a focus on how metabolism is taken into account and a review of class thresholds and the underlying science.

A revised JECFA decision-tree for the evaluation of flavours was proposed. After a brief discussion, the Committee recommended that further considerations are necessary and that a proposal should be drafted for consideration at the next JECFA meeting at which flavouring agents will be evaluated.

The Committee was also informed about a new decision-tree under development by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) for the evaluation of pesticide metabolites using the TTC principle.

2.4 Food additive specifications

2.4.1 Limits for lead in specifications of food additives for use in infant formulas

The Committee at the present meeting considered four additives for use in infant formula and formula for special medical purposes – namely, carrageenan; citric and fatty acid esters of glycerol (CITREM); pectin; and starch sodium octenyl succinate (OSA-modified starch). The Committee noted that the Eighth Session of the Codex Committee on Contaminants in Foods (CCCF) *(7)* agreed to a maximum level (ML) of 0.01 mg/kg for lead in infant formula (as consumed). The Committee also noted that with the exception of carrageenan, use of the other three food additives at proposed use levels could result in an exceedance of the ML of lead in infant formula. This situation was estimated to occur if lead were present in the additive at the specified limit – i.e. 2 mg/kg in CITREM and starch sodium octenyl succinate (OSA-modified starch) and 5 mg/kg in pectin. This estimation was calculated without considering the contribution of other ingredients to the overall lead level in infant formulas.

The Committee noted that the responsibility for ensuring that the final infant formulas comply with the ML for lead remains with infant formula producers. Furthermore, the Committee noted that data provided at the present meeting by the sponsors indicate that individual food additives can be produced with lead levels below the specified limits as listed above. Considering this, the Committee noted that lower lead limits in the specifications – for instance, 0.1 mg/kg for starch sodium octenyl succinate (OSA-modified starch), 1 mg/kg for pectin and 0.5 mg/kg for CITREM – would result in none of the additives exceeding the ML for lead in the final infant formula (i.e. 0.01 mg/kg). The specifications for some of the food additives for use in infant formulas that were considered for safety review at this meeting are also used in the manufacture of other foods. Thus, the Committee agreed that it would be necessary to confirm with manufacturers that the lower lead limits would also be achievable for the intended use of these food additives in products other than infant formulas.

The Committee refers back to CCFA on whether specific purity criteria for additives for use in infant formulas should be considered and appropriate ways to present these criteria (e.g. establishing specifications for additives for use in infant formulas only; establishing different purity limits for additives for use in existing specifications).

As an additional consideration, the Committee noted that if separate specifications for additives in infant formulas were considered necessary, microbiological criteria should also be included.

2.4.2 Method for alginates assay

While reviewing the specifications for gellan gum, the Committee noted that the method of assay referred to the alginates assay method in Volume 4 of the Combined Compendium of Food Additive Specifications (Annex 1, reference *180*), which uses mercury as one of its reagents. The Committee replaced the alginates assay method with a new method, based on the United States Pharmacopeia *(8)*, without the use of mercury. The revised method will be published in the Compendium of Food Additive Specifications, FAO JECFA Monographs 16 (2014).

The online version of the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1, Volume 4, will also be revised.

2.4.3 Oxalate limit test

While reviewing the specifications for citric acid, the Committee recognized that the oxalic acid standard used for comparison in the oxalate limit test, as described in Volume 4 of the Combined Compendium of Food Additive Specifications (Annex 1, reference *180*), does not represent the limit specified in the individual specifications monographs for different food additives. The method was revised to use an appropriate standard solution that represents the limit specified. The revised test method will be published in the Compendium of Food Additive Specifications, FAO JECFA Monographs 16 (2014).

The online version of the Combined Compendium of Food Additive Specifications, FAO JECFA Monographs 1, Volume 4, will also be revised.

2.5 The use of the margin of exposure (MOE) for the evaluation of additives used in infant formulas

The ADI concept does not apply to infants up to the age of 12 weeks because they might be at risk at lower levels of exposure compared with older age groups. This is due to special considerations, such as their immature metabolic capacities, the greater permeability of the immature gut, and their rapid growth and development. Therefore, risk characterization for very young infants has to be considered on a case-by-case basis.

Toxicological testing strategies for additives to be used in infant formulas require approaches that differ from those generally adopted for food additives. For example, evaluation of food additives to be used in infant formulas requires

consideration of safety studies involving exposure of very young animals *(5)*. The reproductive and developmental toxicity studies commonly available for evaluations of chemicals in food address the possible impact on neonatal animals arising through in utero and lactational exposure. However, they frequently do not incorporate direct oral administration to neonatal animals, and such studies are required for the evaluation of food additives in infant formula. If the additive is proposed for use in infant formula at relatively high levels (e.g. 0.1% or greater), then conducting toxicological studies in neonatal animals at doses two or more orders of magnitude greater than the anticipated human exposure, which is the approach commonly taken for food additives, may not be feasible.

The Committee noted that for three of the four food additives on its current agenda that are proposed for use in infant formulas, the margins of exposure (MOEs) between the no-observed-adverse-effect level (NOAEL) and the estimated daily exposures to the food additives were in the range of 0.8–12 for infants. Interpretation of the MOE needs to take into account uncertainties or conservatisms that may exist in the toxicological point of departure or in the exposure estimates.

Considerations related to the toxicological point of departure to be taken into account in interpreting the MOE include:

- absorption, distribution, metabolism and excretion – for example, whether or not the additive is absorbed, comparison of potential for metabolic activation and detoxication in the neonatal organism compared with the adult;
- the overall toxicological profile of the substance, including identification of critical effects;
- the potential effects of exposure during life stages in experimental animals of relevance to human infants;
- the relevance for the human infant of the neonatal animal models used in toxicological testing;
- whether adverse effects have been identified in the toxicological studies in neonatal animals, or if the NOAELs are the highest doses tested;
- the design and outcome of any clinical studies conducted with infants (e.g. total number and age of infants tested, growth, tolerance, types of adverse reaction examined); and
- reports of adverse reactions in post-marketing surveillance, where the infant formula is already in use in some countries.

Factors related to the dietary exposure assessments that should be taken into account for the interpretation of an MOE include the following assumptions and considerations:

- Formula is the only source of nutrition for the first 12 weeks of life.
- The additive will be used at the maximum proposed level.
- An energy density of 67 kcal/100 mL (280 kJ/100 mL) is used to convert energy to the volume of formula ingested daily.
- High infant formula consumption is derived from 95th percentile energy intakes.
- Variability of exposure among infants is small.
- Duration of exposure is for a limited time, and exposure decreases on a body weight basis during the exposure period.

The Committee concluded that when the above issues have been taken into account, an MOE in the region of 1–10 could be interpreted as indicating low risk for the health of infants aged 0–12 weeks consuming the food additive in infant formula.

2.6 Need for an approach for prioritizing flavouring agents for re-evaluation

At this meeting, the Committee held a preliminary discussion concerning the fact that the submission of additional toxicology data, including genotoxicity data, and/or exposure data for new or previously evaluated flavouring agents may trigger the need for re-evaluation of previously evaluated flavouring agents.

Three examples encountered at the present meeting are described. In the first example, 3-acetyl-2,5-dimethylthiophene (No. 1051) was on the agenda for reconsideration at this meeting because new data suggested genotoxic potential. Positive in vitro and in vivo genotoxicity data raised concerns about No. 1051 and previously evaluated thiophenes that are metabolized to thiophene epoxides, indicating that reconsideration of the Committee's conclusions regarding the safety of the previously evaluated thiophenes is warranted.

Second, 2-phenyl-2-butenal (No. 1474) was evaluated earlier as a flavouring agent, and it is structurally related to (±)-2-phenyl-4-methyl-2-hexenal (No. 2069), under consideration at this meeting. Genotoxicity data for No. 1474, used as a structural analogue for No. 2069, were equivocal, raising concerns about the potential genotoxicity of No. 1474 and possibly other previously evaluated compounds with similar structures in this group, in addition to No. 2069. The Committee noted that No. 2069 should not be evaluated for use as a flavouring agent until the concerns related to genotoxicity are resolved, and the safe use

of No. 1474 and structurally related substances as flavouring agents should be reconsidered.

Third, *trans*-α-damascone (No. 2188) was submitted for evaluation at the current meeting of the Committee. Several isomers of No. 2188 were evaluated previously by the Committee, including β-damascone (No. 384), α-damascone (No. 385) and δ-damascone (No. 386), and each was found to be of no safety concern based on dietary exposures estimated by the maximized survey-derived intake (MSDI) method. At this meeting, the same toxicological database used for the evaluation of Nos 384–386 was used for No. 2188. However, the NOAEL for No. 384, used as a structural analogue for No. 2188, was only 200 times the single-portion exposure technique (SPET) estimate for exposure to No. 2188 (600 μg/day). If the SPET estimate of exposure for use of No. 384, No. 385 or No. 386 as a flavouring agent is similar to that for No. 2188, the safety of each of these compounds for use as a flavouring agent could be called into question.

Based on the evaluations conducted on these flavouring agents at the present meeting, the Committee recommended that an approach be developed for prioritizing flavouring agents for re-evaluation based on all available toxicological data and updated exposure estimates.

3. Specific food additives (other than flavouring agents)

The Committee evaluated two food additives for the first time and re-evaluated three others. In addition, the Committee evaluated the safety of four previously evaluated food additives for use in infant formula and formula for special medical purposes intended for infants. Five food additives were considered for revision of specifications only. Information on the safety evaluations and specifications is summarized in Annex 2. Details of further toxicological studies and other information required for certain substances are summarized in section 5.

3.1 Safety evaluations

3.1.1 Benzoe tonkinensis

Explanation

Benzoe tonkinensis is a balsamic resin from the *Styrax tonkinensis* Pierre Craib ex Hartwich tree, which belongs to the Styracaceae family. Benzoe tonkinensis is being evaluated for use as a flavouring agent. The Committee previously considered benzoin gum at its twenty-first, fifty-fifth and seventy-fourth meetings (Annex 1, references *44*, *149* and *205*). At its twenty-first meeting, the Committee prepared tentative specifications covering two forms of benzoin gum (Benzoe tonkinensis and Sumatra benzoin). However, no ADI was established, and no monograph was prepared. At its fifty-fifth meeting, the Committee withdrew the tentative specifications for benzoin gum, as the relevant information was not provided. At its seventy-fourth meeting, the Committee evaluated new information on the composition and the toxicity of Benzoe tonkinensis. Tentative specifications, a Chemical and Technical Assessment and a toxicological monograph were prepared. Comparing the dietary exposure estimate (95th percentile for children) for Benzoe tonkinensis of 0.2 mg/kg body weight (bw) per day with the NOAEL of 500 mg/kg bw per day identified in a 90-day oral toxicity study in rats, the MOE was at least 2500. At the seventy-fourth meeting, the Committee noted that exposure to benzoic acid and benzyl benzoate from the use of Benzoe tonkinensis was well below the upper limit of the group ADI (0–5 mg/kg bw) for benzyl derivatives and that exposure to vanillin was also well below the upper limit of its ADI (0–10 mg/kg bw).

At the seventy-seventh meeting (Annex 1, reference *214*), the Committee revised the specifications, and the tentative status was maintained pending submission of data on microbiological contamination, composition of the ethanolic extract and an analytical method to distinguish between Benzoe tonkinensis and Sumatra benzoin. At the current meeting, additional compositional data were evaluated, and the Committee was able to finalize the safety evaluation.

Chemical and technical considerations

The main compounds identified in Benzoe tonkinensis were benzoic acid, coniferyl benzoate, vanillin and benzyl benzoate. Other compounds, namely *p*-coumaryl benzoate, siaresinolic acid, 3-oxo-siaresinolic acid, sumaresinolic acid and 3-oxo-sumaresinolic acid, were also identified in minor amounts (<1% of the total).

Evaluation

New information on the composition of Benzoe tonkinensis clarified its main constituents. The Committee concluded that dietary exposure to the newly identified compounds (siaresinolic acid, sumaresinolic acid and the respective 3-oxo compounds) would be very low, as their concentrations in Benzoe tonkinensis are very low (<1% of the total).

Given the NOAEL of 500 mg/kg bw per day for Benzoe tonkinensis identified in a 90-day oral toxicity study in rats and the previously established ADIs for the major components, the Committee confirmed the conclusion from the seventy-fourth meeting that Benzoe tonkinensis would not be of safety concern at current estimated dietary exposures, provided that it complies with the specifications prepared at the current meeting, when used as a flavouring agent and in accordance with good manufacturing practice.

The Committee revised the tentative specifications, and the tentative qualification was removed. The definition was amended to identify the main compounds. Microbiological criteria regarding *Salmonella* spp., *Escherichia coli* and yeast and moulds were introduced. The gas chromatographic method for the determination of benzoic acid was updated to allow, besides the determination of benzoic acid and identification of vanillin and benzyl benzoate, the confirmation of the absence of cinnamic acid, which is a marker molecule to differentiate Benzoe tonkinensis from Sumatra benzoin. The existing Chemical and Technical Assessment was revised.

No toxicological monograph was prepared.

3.1.2 Carrageenan

Explanation

Carrageenan is a sulfated galactose polymer with an average molecular weight well above 100 kDa. It is derived from several species of red seaweeds of the class Rhodophyceae. The three main copolymers of carrageenan used in food are designated as iota (ι), kappa (κ) and lambda (λ), depending on the number and location of the sulfate moieties on the hexose backbone. Carrageenan has no nutritive value and is used in foods for its thickening, gelling, stabilizing and glazing agent properties.

Carrageenan was reviewed by the Committee at its thirteenth, seventeenth, twenty-eighth, fifty-first, fifty-seventh and sixty-eighth meetings (Annex 1, references *19*, *32*, *66*, *137*, *154* and *187*). At its twenty-eighth meeting, the Committee established an ADI "not specified" on the basis of the results of a number of toxicological studies on carrageenans obtained from various sources. Processed *Eucheuma* seaweed was reviewed by the Committee at its thirtieth, thirty-ninth, forty-first, forty-fourth, fifty-first and fifty-seventh meetings (Annex 1, references *73*, *101*, *107*, *116*, *137* and *154*). At its fifty-first meeting, the Committee concluded that the toxicities of processed *Eucheuma* seaweed and carrageenan were sufficiently similar for the ADI "not specified" for carrageenan to be extended to a temporary group ADI including processed *Eucheuma* seaweed, pending clarification of the significance of the tumour promotion of known experimental colon carcinogens by carrageenan observed in experiments in rats. At its fifty-seventh meeting, the Committee established a group ADI "not specified" for the sum of carrageenan and processed *Eucheuma* seaweed, as the Committee considered that the intakes of carrageenan and processed *Eucheuma* seaweed from their use as food additives were of no safety concern.

At its sixty-eighth meeting, the Committee reviewed all the available data for toxicological re-evaluation, including specific data relevant to the safety assessment for infants from exposure through infant formula. The previous Committee concluded that potential effects of carrageenan in infants could arise either from a direct action on the epithelium of the intestinal tract, which would be related to the concentration of carrageenan in infant formula, or from absorption of the low molecular weight fraction of carrageenan, which would be more likely to be related to the dietary exposure expressed on a body weight basis. Therefore, the previous Committee estimated MOEs for infants on the basis of both concentration and body weight. In the absence of studies on the immature gut, the Committee used data from studies on adult rodents, which identified the lowest doses causing inflammatory responses in the gut to be in the range 1100–1300 mg/kg bw per day, to derive MOEs. The MOE between the concentration in drinking-water reported to cause inflammation in adult mice and the maximum concentration (0.1%) of carrageenan in infant formula was 10. On a body weight basis, for preweaning infants at the maximum concentration (0.1%) of carrageenan in infant formula, the MOE between the lowest doses reported to cause inflammation in rats and mice and infant exposure was 7; for 12-month-old infants, the MOEs were 50 and 180 at carrageenan concentrations of 0.1% and 0.03%, respectively. The previous Committee considered these MOEs to be insufficient to ensure the protection of infants fed infant formula containing carrageenan and was therefore of the view that it is inadvisable to use carrageenan or processed *Eucheuma* seaweed in infant formula intended for infants up to and including 12 months of age. The group ADI "not specified" for

the sum of carrageenan and processed *Eucheuma* seaweed was maintained for food additive uses in foods other than infant formula.

At the present meeting, the Committee reviewed data published since the sixty-eighth meeting in 2007, focusing in particular on data of relevance to the safety assessment of the use of carrageenan (but not processed *Eucheuma* seaweed) in infant formula. The use levels requested for carrageenan range from 90 to 1000 mg/L, with 300 mg/L as a typical use level for a standard infant formula and higher levels up to 1000 mg/L for liquid formulas for special medical purposes containing hydrolysed protein or amino acids. In response to the Committee's request for further data, a toxicological dossier on carrageenan and a commentary on studies published by an academic research laboratory were submitted. The Committee also considered other information available in the literature of relevance to carrageenan and to the signalling pathways involved in inflammation.

Chemical and technical considerations

Carrageenan is a sulfated galactose polymer, the most important forms being κ-, ι- and λ-carrageenans. κ-Carrageenan (Chemical Abstracts Service [CAS] No. 11114-20-8) is mostly the alternating copolymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose. ι-Carrageenan (CAS No. 9062-07-1) is similar, except that the 3,6-anhydrogalactose is sulfated at carbon two. Between κ-carrageenan and ι-carrageenan, there is a continuum of intermediate compositions differing in degree of sulfation at carbon two. In λ-carrageenan (CAS No. 9064-57-7), the alternating monomeric units are mostly D-galactose-2-sulfate (1,3-linked) and D-galactose-2,6-disulfate (1,4-linked). Various red seaweeds are blended and processed to obtain the desired proportions of κ-, ι- and λ-carrageenan to satisfy food technological requirements. In addition to the polysaccharide components of carrageenan, the product also contains salts (usually potassium chloride to maintain the desired gelling properties) and sugars added for standardization purposes. Other carbohydrate residues (e.g. xylose, glucose and uronic acids) may be present in minor amounts.

Carrageenan has a high average molecular weight distribution of 200–800 kDa, with a small fraction containing naturally occurring fragments in the 20–50 kDa range. A survey of 29 samples of food-grade carrageenan representing κ-, ι- and λ-carrageenan determined a number average molecular weight of 193–324 kDa and a weight average molecular weight of 453–652 kDa. Food-grade carrageenan has a viscosity specification of not less than 5 cP at 75 °C (1.5% solution), which corresponds to an average molecular weight of approximately 100–150 kDa.

Poligeenan (CAS No. 53973-98-1) has been used in research studies as a surrogate for the low molecular weight fraction of carrageenan. It is produced under severe acid and high-temperature conditions and has an average molecular weight of 10–20 kDa. Poligeenan is distinct from food-grade carrageenan. Although the average molecular weights of carrageenan and poligeenan are significantly different, when the molecular weight distributions of the two substances are compared, there is a small overlap between the two at the lower molecular weight portion of the carrageenan distribution and the higher molecular weight portion of the poligeenan distribution, with the range of interest being between 20 and 50 kDa.

When carrageenan is present in water at less than about 0.1% with no dietary solids or protein, the carrageenan molecules are random open coils and available for maximum interaction with other molecules. κ-Carrageenan in the presence of potassium cations and ι-carrageenan in the presence of calcium cations will gel at concentrations above 0.1% in water if a heating/cooling cycle is applied. This requires heating to above about 60 °C, at which temperature the carrageenan is in a random open coil. On cooling to less than about 35 °C, the solutions will gel, forming double helices; then, for κ-carrageenan only, these helices will aggregate and form a more tightly closed structure. The transition between sol (random open coil) and gel (organized closed structure) for κ- and ι-carrageenan is not sharp, but is a progressive continuum from 100% sol to 100% gel, as either carrageenan concentration or gelling cations or both are increased. λ-Carrageenan is non-gelling at all concentrations and cation balances and is therefore always in the random open coil form in water. λ-Carrageenan normally occurs as a minor component in combination with κ-carrageenan in commercial products and enhances the gelling matrix through physical (void filling) and chemical (hydrogen-bonded helix cross-linkages) means. Carrageenan gels do not melt until temperatures are well above 37 °C.

The stability of carrageenan in foods is influenced by several factors, such as pH, direct structural bridging between the negatively charged carrageenan and positively charged protein sites, and indirect structural bridging with negatively charged protein sites via divalent cations such as calcium, through hydrogen bonding and through carrageenan–carrageenan helical interactions. Carrageenan is added to infant formula in order to stabilize the emulsion of protein, fat and water so as to maintain the consistency of the infant formula throughout storage and feeding.

Toxicological data

The form and stability of carrageenan in test materials and in foods are important considerations, both for interpretation of in vitro and in vivo experimental studies

on carrageenan and for assessment of the safety-in-use of carrageenan in a food product such as infant formula. Form and stability can be influenced by the preparation of the food and the nature of the matrix (solid or aqueous, presence or absence of protein) in which it is administered and by its passage through the gut. In previous studies, oral toxicity has been considerably influenced, not only by dose, but also by whether the test material administered was food-grade carrageenan or poligeenan. However, for some studies, the information on the specifications of the test material was inadequate, and sometimes no distinction was made between poligeenan and carrageenan in discussion and interpretation of results. Further, even if the type of carrageenan used is identified as commercial pure λ-, κ- or ι-carrageenan, analyses have shown that test materials may not be as described by the commercial supplier; the test materials may, for example, contain more than one type of carrageenan together with a substantial percentage of sucrose or dextrose.

Earlier studies on absorption in adult rats, guinea-pigs and primates have shown that little or no food-grade carrageenan is absorbed following oral exposure. At previous meetings, the Committee has commented that high molecular weight carrageenan is probably not absorbed. At the sixty-eighth meeting (Annex 1, reference *188*), it was noted that no studies were available addressing the effects of carrageenan on the immature gut, and it was not possible to draw conclusions on whether carrageenan might be absorbed from the immature gut.

For the present meeting, new data on absorption were available from good laboratory practice (GLP)–compliant studies in neonatal minipigs and pigs in which food-grade carrageenan was given in infant formula adapted for pig requirements. These studies used a liquid chromatography/tandem mass spectrometry analytical method that had been developed using poligeenan as a surrogate for detection of the low molecular weight tail of carrageenan in blood. In the minipig study, essentially no signal from low molecular weight carrageenan was detected in serum following oral administration of daily carrageenan doses of 0, 300 or 3000 mg/kg formula for 10 days starting on postnatal day 2, the higher dose being equal to 600–666 mg/kg bw per day. In the pig study, carrageenan doses of 0, 300, 1000 or 2250 mg/kg formula were given for 28 days starting on postnatal day 3, the highest dose being equal to 430–448 mg/kg bw per day. A positive signal corresponding to that expected for the low molecular weight tail of carrageenan was found in male piglets. However, the positive signal was observed in both treated and control males, irrespective of whether they had been given control formula or formula containing carrageenan. The range of values in control and carrageenan-treated males was similar and was not dose related. The signal was not seen in the female piglets, and it is likely that the signal corresponded to some other constituent of male serum, but this was not further investigated or identified.

The Committee concluded that absorption studies in neonatal minipigs and pigs are an appropriate model for the immature gut in human infants. However, because of the problems with the outcomes of the assay, the Committee was unable to conclude that fragments in the low molecular weight tail of carrageenan (molecular weight range 20–50 kDa) are not absorbed across the immature gut into the systemic circulation.

The suitability of carrageenan for use in infant formula requires additional considerations to those for the general use of carrageenan in foods. Of the information reviewed previously by the Committee, all animal experiments, apart from one in infant baboons given infant formula containing carrageenan, were performed in adult animals, which limits their usefulness for the safety evaluation of carrageenan for infants. From the absence of effects in the study in infant baboons, a no-observed-effect level (NOEL) of 1220 mg/L in formula was identified, equivalent to an exposure of 432 mg/kg bw per day. However, as the previous Committee commented at its sixty-eighth meeting (Annex 1, reference *188*), in this study, the colon was fixed in 10% buffered formalin, and this does not enable identification of mast cells that would be present if an inflammatory process had been initiated.

In the new GLP-compliant toxicological studies in neonatal minipigs and pigs that were submitted for this meeting, there was extensive microscopic study of sections taken from along the length of the intestine and the use of appropriate fixatives and stains for visualization of mucosal mast cells. Additionally, in the pig study, goblet cells in the villi and crypts of the jejunum were examined by appropriate staining. Immunophenotyping of leukocytes, measurement of proinflammatory cytokines interleukin-1 (IL-1), IL-6, IL-8 and tumour necrosis factor alpha (TNFα) in blood, and immunohistochemistry of the gut to assess the presence of IL-8 and TNFα were also carried out.

In the 10-day study in neonatal minipigs, no treatment-related effects were observed, apart from reduced feed consumption and reduced body weight gain on some days in male and female minipigs given the highest carrageenan concentration of 3000 mg/kg formula. Increased red blood cell count, haemoglobin and haematocrit were also seen in males given a carrageenan concentration of 3000 mg/kg formula, which may have been related to the reduced consumption of formula in the high-dose group. Detailed microscopic assessment of the gastrointestinal tract showed no evidence of inflammation or other lesions, and there was no effect of carrageenan treatment on mucosal mast cell counts. The NOAEL for carrageenan from this study was 300 mg/kg formula, equal to an exposure of 74 mg/kg bw per day, but it should be noted that the effects seen at the higher dose were likely attributable to reduced palatability and the reduced feed consumption at that dose.

In the 28-day neonatal pig study, in which the highest carrageenan concentration of 2250 mg/kg formula was less than the highest concentration in the minipig study, there were no treatment-related effects on body weight or feed consumption. There were also no treatment-related effects on haematology, clinical chemistry, organ weights, or organ and tissue histopathology. The extensive microscopic assessment of the gastrointestinal tract showed no evidence of inflammation or other lesions, and there was no effect of carrageenan treatment on mucosal mast cell counts. Similarly, there were no treatment-related effects on immunophenotyping of blood lymphoid subsets or proinflammatory cytokines in the blood or in the gut. The NOAEL for carrageenan from this study was 2250 mg/kg formula, equal to an exposure of 430 mg/kg bw per day.

The Committee considered that the neonatal pig and minipig studies in which formula containing carrageenan was given during the first month of life were appropriate to model the human infant from 0 to 12 weeks of age, when infant formula may be provided as the sole source of nutrition. It is also a relevant model for the decrease in permeability of the gut epithelium to macromolecules during the neonatal period ("gut closure") and immunological development. The type of carrageenan administered (predominantly κ-carrageenan) and the use of adapted infant formula as the matrix for the studies also adequately reflected the types of infant formula containing carrageenan available for human infants. The Committee noted that although these are liquid rather than solid foods, infant formula contains proteins that bind carrageenan. The pig and minipig studies have provided considerable information to address the issues of whether inflammation occurred in neonatal animals and whether any damage was done to the gut mucosa. No adverse effects were observed on the gut or on the immune parameters assessed. The Committee concluded that the NOAEL from the neonatal pig study could be used to estimate MOEs for human infants consuming formula containing carrageenan.

The recent in vitro studies on inflammatory pathways reviewed at this meeting and the results of some of the studies reviewed by the Committee at previous evaluations raise the question of whether carrageenan might play a role in intestinal inflammation. The Committee agreed with the problems that have been pointed out by others in some of the methodological aspects of the in vitro studies reviewed at this meeting. In addition, the Committee noted that the in vitro studies with carrageenan were not validated by assessment of responses to a positive control, such as a known inflammatory substance. The Committee also had concerns about the use of proliferating cell lines, as inflammatory effects in the gut would be expected to be exerted on the mucin-producing cells at the tips of the intestinal villi and not on the proliferating cells in the crypts. More importantly, the Committee considered that there are also difficulties in extrapolating findings from in vitro studies on human intestinal cell cultures to draw conclusions on risk

assessment for humans in vivo. This aspect is particularly relevant given that in vitro systems reflect only one component of the in vivo processes for prevention of gut inflammation, which are known to be complex.

It is also evident that the observations on activation of inflammatory or mitogenic signalling pathways in human colonic cells cultured in vitro are in contrast to findings from in vivo studies in which carrageenan has been given in the diet. There is no evidence of intestinal inflammation or lesions in laboratory animal studies in which well defined, undegraded carrageenan, which nevertheless has a small proportion of lower molecular weight components, has been given orally in the diet, in contrast to some results from administration in the drinking-water. There may be several reasons for this, including that in vivo the cells lining the gastrointestinal tract are protected by a mucous barrier that is not present in in vitro models and that dietary administration, as opposed to drinking-water administration, offers the opportunity for carrageenan to bind to protein.

Human studies

The Committee noted that only two brief reports of studies on infants given formula containing carrageenan are available. Both were conducted in healthy newborn infants. In one 6-month study, 1269 infants were given infant formula containing carrageenan at 300 mg/L and compared with 149 infants given formula with no carrageenan. In the other 112-day study, 100 infants were fed formula containing carrageenan at 1000 mg/L and compared with 95 infants given formula with no carrageenan; 58 infants failed to complete the trial, but the numbers dropping out due to intolerance were similar in both groups. Detailed descriptions of these studies were not available, and the brief reports did not indicate any health problems in either study.

Assessment of dietary exposure

The typical level of carrageenan used in reconstituted powdered and liquid cow milk– and soy-based formulas is 0.009–0.1 g/100 mL (90–1000 mg/L), with the higher levels being used in formulas containing hydrolysed proteins. Current use levels include 0.03 g/100 mL (300 mg/L) for regular milk- and soy-based liquid formulas and 0.1 g/100 mL (1000 mg/L) for hydrolysed protein– and/or amino acid–based liquid formulas.

Median infant formula consumption estimates were derived from estimated energy requirements for fully formula-fed infants. It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. A further exposure scenario was considered, using high (95th percentile) daily energy

intakes reported for formula-fed infants. The highest reported 95th percentile energy intakes were for infants aged 14–27 days. For all dietary exposure estimates, a common energy density of formula of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy to the volume of formula ingested daily.

For infants aged 0–6 months, median dietary exposure to carrageenan was estimated to be in the range 37–54 mg/kg bw per day for carrageenan added to infant formula at 300 mg/L formula, as consumed. At a use level for carrageenan of 1000 mg/L formula, as consumed, median estimated dietary exposure was in the range 124–180 mg/kg bw per day. High (95th percentile) exposures to carrageenan were estimated to be 65–67 mg/kg bw per day for a use level of 300 mg/L and 218–222 mg/kg bw per day for a use level of 1000 mg/L.

Evaluation

New studies relevant to the evaluation of the use of carrageenan in infant formula and formula for special medical purposes have been conducted since the Committee last considered this issue (Annex 1, references *187* and *188*). They include investigations of absorption and toxicity in both the neonatal minipig and neonatal pig in which carrageenan was fed in infant formula adapted for piglets.

The absorption studies did not allow any conclusions to be reached. In the toxicity studies, in addition to a wide range of toxicological parameters, a detailed examination of the histology of all segments of the gastrointestinal tract and quantification of mast cells along the gastrointestinal tract were undertaken in both the minipig and pig. In the pig, an appropriate array of serum and gut cytokines was also assessed, together with blood leukocyte immunophenotyping. From these new investigations, there was no evidence of any inflammation in the gut or any effects on immune parameters. A NOAEL of 430 mg/kg bw per day, which was the highest dose tested, was derived from the neonatal pig study. The Committee also noted that the NOAEL of 430 mg/kg bw per day from the neonatal pig study is almost identical to that from the earlier infant baboon study of 432 mg/kg bw per day.

In the 10-day neonatal minipig study, animals were given infant formula containing carrageenan at concentrations up to 3000 mg/kg (0.3%). Concentrations of carrageenan above approximately 2500 mg/kg (0.25%) become highly viscous, and this appears to have adversely affected palatability and growth in the minipigs. Accordingly, the amount of carrageenan added to the formula fed to piglets in the main study was reduced to 2250 mg/kg (0.225%). As a consequence of this limitation, the MOEs between the NOAEL from the pig study and human infant exposures at 2–4 weeks of age range from 2 to 12 on a body weight basis and from 2 to 8 on a concentration basis.

The Committee noted that although the MOEs are small in magnitude (see section 2.5), they are derived from a neonatal pig study in which the highest dose tested was without adverse effects on the gut or on immune parameters, supported by a neonatal minipig study. The neonatal pig and minipig are appropriate models for the young human infant up to at least 12 weeks of age, for whom infant formula may be the sole source of nutrition. These new studies allay the earlier concerns that carrageenan, which is unlikely to be absorbed, may have a direct effect on the immature gut. The Committee also took account of the previous toxicological database on carrageenan, which did not indicate other toxicological concerns.

The Committee concluded that the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1000 mg/L is not of concern. The Committee recognizes that there is variability in medical conditions among infants requiring formulas for special medical purposes that contain the higher levels of carrageenan, and the Committee notes that these infants would normally be under medical supervision.

The Committee at its sixth-eighth meeting (2007) had prepared specifications for carrageenan. The Committee discussed limits on lead specifications for this and the other food additives for use in infant formulas that were on the agenda, as described in section 2.4.1. The Committee revised the specifications with minor changes.

An addendum to the toxicological monograph was prepared.

3.1.3 Citric and fatty acid esters of glycerol (CITREM)

Explanation

Citric and fatty acid esters of glycerol (CITREM) are listed in the GSFA *(3)* for use as an antioxidant, emulsifier, flour treatment agent, sequestrant and stabilizer in several food categories.

The Committee previously reviewed CITREM at its seventeenth (1973), thirty-fifth (1989) and sixty-first (2003) meetings (Annex 1, references *32*, *88* and *166*). At its seventeenth meeting, the Committee allocated an ADI "not specified" to CITREM. The Committee based its safety evaluation on biochemical and metabolic studies demonstrating that this substance is completely hydrolysed in the gastrointestinal tract into components that are normal constituents of the diet, together with knowledge of the metabolism and lack of toxicity of citric acid, glycerol and fatty acid esters of glycerol.

At the request of CCFA at its Forty-fifth Session *(9)*, the Committee evaluated the safety of CITREM for use as an emulsifier in infant formula and formula for special medical purposes intended for infants, to replace the combined use of three emulsifiers – lecithin, monoglycerides and diglycerides

of fatty acids, and diacetyl tartaric acid ester of monoglycerides and diglycerides. The proposed use levels considered at this meeting were up to 7.5 g/L as consumed in reconstituted infant formula powder and up to 9 g/L in ready-to-feed liquid infant formula. The higher amounts are used in formulas based on amino acids or (partially) hydrolysed protein.

Chemical and technical considerations

CITREM (INS 472c) is a white to ivory coloured, oily to waxy material. It is a mixture of citric acid esters and fatty acid esters of glycerol and is obtained by esterification of glycerol with citric acid and food-grade fatty acids or by reaction of a mixture of monoglycerides and diglycerides of food-grade fatty acid with citric acid. The structural formula for CITREM is shown below:

$$\begin{array}{l} CH_2-OR_1 \\ | \\ CH-OR_2 \\ | \\ CH_2-OR_3 \end{array}$$

where at least one of R_1, R_2 or R_3 represents a citric acid moiety or a fatty acid moiety and the remainder may represent citric acid, fatty acid or hydrogen. CITREM is mainly composed of glycerol (8–33%), fatty acids (37–81%) and citric acid (13–50%) and could contain up to 4% of free glycerol, minor amounts of free fatty acids, free citric acid, and monoglycerides and diglycerides. CITREM may be wholly or partially neutralized with sodium hydroxide or potassium hydroxide.

Biochemical data

A newly available in vitro study on the digestibility of CITREM itself and CITREM-containing infant formula confirms that hydrolysis of CITREM by gastric and pancreatic lipases occurs under conditions in which the pH and amount of bile salts are varied to mimic those in the stomach and duodenum of term and preterm human infants. However, in this two-stage in vitro model, when CITREM in infant formula was added, hydrolysis of CITREM into its component parts of glycerol, citric acid and fatty acids was incomplete and in the range of 14–28%; this was lower than expected. The likely reason for the partial hydrolysis was shown in further studies in the two-stage in vitro model in which hydrolysis of pure citric acid and fatty acid esters of glycerol (i.e. the main components of CITREM without any free glycerol, free citric acid, free fatty acids or free glycerides) was found to be around 2-fold higher (47–58%) than for CITREM in infant formula. This confirmed the suspected negative effect of free glycerides on the action of lipases. These data suggest that, in vivo, hydrolysis of

CITREM is likely to continue lower down the small intestine as the glycerides and free fatty acids from breakdown of the fats in infant formula and CITREM form micelles and are progressively absorbed by the enterocytes. Absorption of the contents of the micelles occurs mainly in the proximal jejunum and partly in more distal segments of the small intestine, which are not modelled in the two-stage in vitro model. The model also did not include lingual lipase, which would contribute to overall hydrolysis in vivo.

Toxicological data

There are few toxicological studies available on CITREM from previous evaluations. The only new information available on CITREM for this evaluation is a report that an in vitro study on *Salmonella typhimurium* did not show any evidence of gene mutations and a short-term study on the effects of CITREM on fat absorption in the rat, which was not considered useful for the evaluation because of the very high amount of CITREM used.

The Committee considered the hypothesis, based mainly on in vitro studies, that food emulsifiers may decrease the integrity of the intestinal epithelial barrier. The evidence for such a hypothesis is limited, particularly because in the in vitro studies, surfactants and emulsifiers have been applied directly to cells at concentrations (e.g. 1 mg/mL) that are likely to exceed those occurring normally in the gut lumen following consumption of foods containing emulsifiers. It should also be noted that monolayers of a human colon cancer–derived cell line (CaCo2) used in these studies do not mimic physiological conditions; for example, they lack goblet cells that secrete mucin, so they do not have the protective layer of intestinal mucus that would be present in vivo. None of the in vitro studies used CITREM. Several studies using emulsifiers with significantly higher hydrophilic–lipophilic balance (HLB) values than those of CITREM showed disruption of epithelial integrity. Although the surfactant activity of food emulsifiers demonstrated in in vitro models makes it relevant to consider this hypothesis, it is necessary to take account of the differences between normal physiological conditions in the gut during food digestion, including the amounts of emulsifier present, the hydrolysis and dilution of the emulsifier, and the conditions of the experimental studies, before concluding on relevance for health. It is not possible to conclude from the studies conducted to date on emulsifiers other than CITREM, with higher HLB values than those of CITREM, that CITREM itself will affect the intestinal barrier under in vivo conditions.

Human studies

Tolerance of infants to formulas containing CITREM at concentrations ranging from 0.95 to 1.62 g/L is supported by a number of clinical studies. Similarly, a

formula containing added citric acid salt at 2.5 g/L was also well tolerated by infants. No clinical trials on tolerance of infants to formulas containing higher amounts of CITREM, up to the maximum of 9 g/L used in some formulas for special medical purposes intended for infants, have been submitted to the Committee. The Committee was provided with a summary of five case reports on infants aged 2–11 months given a liquid, peptide-based formula containing a high concentration of CITREM (8.56 g/L) for 2 or 4 weeks. These did not allow any conclusions to be drawn on tolerance, as all the infants had pre-existing gastrointestinal disorders or diseases; some additionally had other health problems; and some had loose or soft stools before starting on the formula.

The Committee considered whether there may be adverse effects from citric acid released in the gut from CITREM. Citric acid has been evaluated previously by the Committee (Annex 1, reference *33*) and given an ADI "not limited",[3] but this evaluation did not cover infants less than 12 weeks of age. The estimated total exposure of infants in the age range 14–27 days (the age range with the highest exposures) to citric acid, derived from both the natural constituents of formula and CITREM added to formula at 9 g/L, is up to 930 mg/kg bw per day for infants with median energy requirements and up to 1140 mg/kg bw per day for infants at the 95th percentile energy intake, if citric acid is assumed to be present at the upper end of the range reported for CITREM (13–50%).

Oral rehydration solutions delivering citrate at about 98 mg/kg bw per day were without adverse effects in a double-blind randomized control study in infants and children. A study in low birth weight infants, preterm infants and term infants on the effect of supplementation of cow's milk formula with citrate salts, giving doses of about 500 mg/kg bw per day, for 3 weeks from birth reported no adverse effects on growth or tolerance, including stool frequency, compared with controls not receiving citrate. However, in a small study in which free citric acid was given in divided doses over 24 hours, diarrhoea occurred in four of eight infants aged 4–12 months receiving total amounts (food citrate plus free citric acid) equivalent to a citric acid exposure of approximately 400–700 mg/kg bw per day. The Committee noted that citric acid–based oral rehydration solutions in which trisodium citrate (2.94 g/L) or tripotassium citrate (3.24 g/L) is used to replace bicarbonate have been successfully used in the treatment of diarrhoea in infants and adults. Oral sodium or potassium citrate, taken as tablets or in solution in divided doses, is also prescribed to alkalinize the urine for the treatment of urinary tract infections, hypocitraturia and kidney stones, including at doses of 1–4 mEq/kg bw per day (108–430 mg/kg bw per day) in infants and children. Diarrhoea is listed as an occasional side-effect of potassium citrate treatment, due to the irritant effect in the gut. It is also noted that formula of a similar composition to some formulas for

[3] A term no longer used by JECFA that has the same meaning as ADI "not specified".

special medical purposes intended for infants, but not containing CITREM, has been associated with softening of stools in infants.

Assessment of dietary exposure

Dietary exposures were estimated for a typical use level of CITREM of 2.7 g/L in powdered infant formula after reconstitution and the upper range of requested use levels of CITREM at 7.5 g/L and 9 g/L, for use in formula for special medical purposes intended for infants. For CITREM subcomponents, it was assumed that complete hydrolysis into its free components would occur. Of the components of CITREM, only citric acid was identified as requiring an exposure estimate from consumption of formula containing CITREM. It has been reported that CITREM may contain 13–50% citric acid. Basal levels of citric acid in "typical" prepared infant formulas (0.64 g/L) were also considered in the assessment of exposure to citric acid.

Median infant formula consumption estimates were derived from estimated energy requirements for fully formula-fed infants. It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. A further exposure scenario was considered, using high (95th percentile) daily energy intakes reported for formula-fed infants. The highest reported 95th percentile energy intakes were for infants aged 14–27 days. For all dietary exposure estimates, a common energy density of formula of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy to the volume of formula ingested daily.

For infants aged 0–6 months and a 2.7 g/L CITREM use level, median estimated exposures to citric acid are in the range 120–360 mg/kg bw per day, with the high end of the range relating to infants 0–1 month of age and a citric acid content for CITREM of 50%. At the high end of intended use (9 g/L), the median citric acid exposure estimates are in the range 230–930 mg/kg bw per day. For 95th percentile consumers 14–27 days of age and considering a 2.7 g/L CITREM usage, estimated citric acid exposures are up to 440 mg/kg bw per day; at the higher requested use level (9 g/L), the 95th percentile citric acid exposure estimates are up to 1140 mg/kg bw per day for a citric acid content for CITREM of 50%.

Assuming that CITREM would have an energy density equivalent to that of triglycerides (9 kcal/g), it has been estimated that CITREM is a low contributor to energy intakes (3.6–12.1%), depending on the use level.

Evaluation

The new study on CITREM digestibility in a two-stage in vitro model mimicking the stomach and duodenum of preterm and term infants showed incomplete hydrolysis of CITREM. However, the model did not simulate the entire digestion

process or the further hydrolysis that is likely to occur lower down the gut. The Committee concluded that CITREM was likely to be substantially hydrolysed in the gut in vivo and that any partially hydrolysed products, such as glycerol citric acid esters, would not be of safety concern.

The Committee considered the limited available evidence on whether free citrate in the gut would cause diarrhoea. In one study, no effects were observed in 13 infants exposed to free citrate, given as citrate salts added to formula, at 500 mg/kg bw per day. In another study, diarrhoea was observed in four out of eight infants given free citric acid by gavage in divided doses over 24 hours, equivalent to a total exposure to free citrate of approximately 400–700 mg/kg bw per day. The Committee noted that the diarrhoea may have been due to osmolality and the gavage mode of administration. In the gut, the enzymatic release of free citrate from infant formula containing CITREM would be more gradual. The Committee was also aware that citrate salts have been used in oral rehydration solutions for the treatment of diarrhoea in infants. Clinical trials in infants show tolerance to formulas containing CITREM at up to 1.6 g/L, but there are no tolerance trials in infants given formula containing CITREM at the high end of the requested range (9 g/L).

Taking the above considerations into account, it is unlikely that consumption of formulas containing typical levels of CITREM used in powdered formulas (up to 2.7 g/L as reconstituted), which is equivalent to an exposure to citrate of 440 mg/kg bw per day for the very young infant at the 95th percentile energy intake, would cause diarrhoea. At the high end of the requested range (up to 9 g/L), which is equivalent to an exposure to citrate of 1140 mg/kg bw per day for the very young infant at the 95th percentile energy intake, diarrhoea might occur in some infants.

The Committee concluded that there are no toxicological concerns about the use of CITREM in infant formula and formula for special medical purposes at concentrations up to 9 g/L. At the higher use levels, there is a possibility of diarrhoea from free citric acid released from formula containing CITREM. Given the paucity of clinical data and the fact that exposure assumptions for citric acid have been maximized, it is difficult to estimate the risk of diarrhoea, but it is considered to be low.

At the present meeting, the Committee reviewed the specifications for CITREM. The Committee discussed limits on lead specifications for this and the other food additives for use in infant formulas that were on the agenda, as described in section 2.4.1. The Committee also noted that the test method for the determination of total citric acid currently employs a gas chromatographic method using a packed column. The Committee recommends replacing this method with a suitable method using a capillary/wide-bore column, for consideration at a future meeting.

Based on the information available, the Committee revised the existing specifications, making minor changes to the purity tests for CITREM.

An addendum to the toxicological monograph was prepared.

3.1.4 Gardenia yellow

Explanation

Gardenia yellow is an extract from the fruit of *Gardenia jasminoides* Ellis that is used as a food colour in some countries. The main colouring principals of gardenia yellow are crocetin and crocin. Crocetin and crocin also occur in saffron, which was evaluated by the Committee at its fourth, twenty-first and twenty-ninth meetings (Annex 1, references *4*, *44* and *70*). Saffron was regarded as a food rather than a food additive.

Gardenia yellow has not been evaluated previously by the Committee. It was on the agenda at the request of the Forty-fifth Session of CCFA *(9)*.

Chemical and technical considerations

Gardenia yellow is produced by ethanol extraction from the fruits of *Gardenia jasminoides* Ellis and subsequent purification. The colouring principals of gardenia yellow are the carotenoid crocetin and crocetin esters. Crocin (crocetin di-gentiobiose ester) is the major crocetin ester. Geniposide, a substance with reported therapeutic effects, may also be present in the final product at up to 0.5%.

The Committee was unable to evaluate the chemical characteristics of the product owing to the lack of relevant data and because of inconsistent and contradictory information provided by the two sponsors.

Biochemical data

The available data show that when given orally as a single dose, crocin, the main component of gardenia yellow, is not absorbed. Deglycosylation of crocin in the intestinal tract produces crocetin, which is then rapidly absorbed and distributed. It is claimed in the submission that crocetin is absorbed following the same pathway as for other carotenoids (e.g. β-carotene, lutein, lycopene). Evidence was provided that absorbed crocetin is partly metabolized to monoglucuronide and diglucuronide conjugates in mice and has an elimination half-life of about 7 hours in humans.

Toxicological data

No acute toxicity was reported in studies with mice and rats at doses of gardenia extract (composition unknown) up to 20 g/kg bw and 4 g/kg bw, respectively. Mice received a single intragastric administration of an aqueous solution of gardenia yellow powder at a dose of 10, 15 or 20 g/kg bw. This powder was

described by the authors as containing 92% of a gardenia extract with crocin as the main component (the method of preparation was not given). Rats received a single intragastric administration of either a suspension in water of the same preparation of gardenia yellow powder (females: 3 g/kg bw; males: 4 g/kg bw) or a water extract of gardenia yellow of unknown preparation and composition (3 g/kg bw for both males and females).

In a 13-week study, rats were fed gardenia yellow extract at a concentration of 0, 750, 1500 or 3000 mg/kg diet (equivalent to 0, 75, 150 and 300 mg/kg bw). Gardenia yellow intakes were calculated by the authors as 0, 35, 72 and 143 mg/kg bw per day for males and 0, 43, 88 and 166 mg/kg bw per day for females, respectively. The gardenia yellow powder added to the diet was described as containing 92% of a gardenia extract, with crocin being stated as the main component. A NOAEL for gardenia yellow of 72 mg/kg bw per day, corresponding to 1500 mg/kg in the diet, was identified by the authors, based on increased serum aspartate transaminase activity together with an accumulation of lipid droplets in the hepatocytes seen at 3000 mg/kg diet, the highest concentration tested. The Committee noted that the exact composition of the material tested was not described, the weights of the animals at the end of the study and the feed consumption were not provided, and details on the observed effects were not reported.

In another 13-week study, no effects were reported in rats with dietary exposure to gardenia yellow equivalent to 300 mg/kg bw per day. The Committee noted that the composition of the gardenia yellow used was not known.

No long-term toxicity or carcinogenicity studies were available.

Gardenia yellow (composition unknown) was tested in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 using the plate incorporation and the preincubation methods. In the preincubation test, the concentrations of gardenia yellow were 10, 25 and 50 mg/mL. No genotoxicity was reported. Another Ames test with concentrations of gardenia yellow (composition not given) up to 50 mg/plate was also negative, with and without metabolic activation.

In V79 cells, gardenia yellow (15.6–1000 μg/mL of a water-soluble extract from *Gardenia jasminoides*) caused DNA damage in the rec-assay at all the concentrations tested and induced a significant dose-dependent increase in sister chromatid exchange frequency. Three-dimensional capillary electrophoresis analysis of the extract did not show any genipin (a substance with known genotoxic potential formed by hydrolysis of geniposide), and there was only one peak, which was considered by the authors to correspond to geniposide. Geniposide did not show any genotoxic activity in this study. The Committee noted that the compound responsible for the genotoxic effect was not identified.

In an in vivo bone marrow micronucleus test, mice received a single dose of gardenia yellow (composition unknown) in aqueous solution by gavage at 2.5, 5 or 10 g/kg bw. No genotoxic effects were seen.

No multigeneration reproductive toxicity studies were available.

Mice receiving an aqueous solution of gardenia yellow (composition unknown) by gavage for 5 consecutive days at a dose of 2.5, 5 or 10 g/kg bw per day were terminated 35 days after the first day of treatment. No morphological abnormalities were reported in the sperm of the mice of the lower two dose groups. All animals that received 10 g/kg bw per day for 5 days died before the end of the study.

No developmental toxicity studies were available, with the exception of a study using *Xenopus* embryos. A teratogenic potential for crocetin was reported. The Committee considered this study to be not relevant for its evaluation of gardenia yellow.

Several special studies were presented in the submission. The Committee noted that most of these studies were not designed to evaluate potential adverse effects. In addition, the studies used saffron as the source of crocin, and most of them described therapeutic effects of purified crocin. Therefore, the Committee considered these studies to be not relevant for its safety evaluation of gardenia yellow.

Two clinical trials in humans were reported. In a double-blind, placebo-controlled, three-way crossover study designed to examine the effects of crocetin on physical fatigue compared with placebo, 14 healthy volunteers (seven men and seven women) were randomized to daily oral administration of capsules containing 15 mg crocetin or placebo for 8 days. The Committee noted that the study was not designed to evaluate adverse effects.

In order to investigate the effect of crocetin (extracted from gardenia yellow; chemical analysis not presented) on sleep, a clinical trial was undertaken comprising a double-blind, placebo-controlled crossover trial of 21 healthy adult men with a mild sleep complaint. Crocetin was administered at 7.5 mg/day in a gelatine capsule during two intervention periods of 2 weeks separated by a 2-week washout period. Body weight, blood pressure and pulse rate, as well as haematological and biochemical analysis of blood samples, did not show significant differences after intake of crocetin compared with placebo.

The Committee noted that the usefulness of these clinical trials was limited for the evaluation of gardenia yellow, as they were of short duration and the administered doses were low.

No epidemiological studies were reported.

Assessment of dietary exposure

Owing to the uncertainties in the composition of the gardenia yellow used, the Committee did not perform an exposure assessment. Uses and use levels in foods are proposed in the submission, and the resulting daily exposures were calculated by the sponsor based on national food consumption data for the Chinese population. Average exposure estimates ranged from 5.5 to 18 mg/kg bw per day for the populations of "females over 18 years" and "1–3 years", respectively. At the 95th percentile, the dietary exposures ranged from 22.8 to 76.9 mg/kg bw per day for these populations.

Evaluation

The Committee noted that the manufacturing process and composition of gardenia yellow were insufficiently described.

The Committee also noted that some studies included in the submission were performed using extracts from *Crocus sativus*, and the possible similarities and differences with the food colour prepared from *Gardenia jasminoides* were not documented. In many of the available studies, the material tested was not well characterized and/or not adequately described. Therefore, it is not clear whether the material tested toxicologically was representative of gardenia yellow.

The Committee further noted that the available toxicity studies have not been conducted following internationally recognized guidelines and that a number of studies were performed using non-relevant (intraperitoneal or intravenous) routes of administration. There are no long-term toxicity, carcinogenicity, reproductive toxicity or developmental toxicity studies available.

Because of the inconsistent and contradictory data provided by the two sponsors, the Committee was unable to prepare a specifications monograph.

In order to establish specifications, the Committee requires:

- information on the manufacturing process, including purification steps;
- analytical data on the composition of the substance, including the total amount of colouring matter and relevant compounds of known biological activity, such as geniposide and genipin;
- data on loss on drying;
- information on a method of assay;
- analytical data on at least five different batches of commercial materials supporting the specifications; and
- data on stability in food.

Given the deficiencies in the toxicological database, including missing toxicological studies (e.g. long-term toxicity, carcinogenicity, reproductive toxicity and developmental toxicity studies), the inadequate characterization of the test material and limited reporting of the available studies, the Committee was unable to evaluate gardenia yellow proposed for use as a food colour.

A toxicological monograph was prepared.

3.1.5 Lutein esters from *Tagetes erecta*

Explanation

Lutein esters contain lutein, (all-*E*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol, a naturally occurring xanthophyll pigment. Lutein occurs with its isomeric xanthophyll zeaxanthin in many foods, particularly vegetables and fruit. Extracts containing xanthophylls (free and/or esterified) are used as colours and as nutritional supplements in a wide range of applications.

Products extracted from *Tagetes erecta* containing lutein and its esters have been the subject of previous JECFA evaluations. At its thirty-first meeting (Annex 1, reference *77*), the Committee prepared tentative specifications for xanthophylls obtained from *Tagetes erecta* petals, but no toxicological data were available, and no toxicological evaluation was performed. *Tagetes* extract containing lutein esters at low concentrations was considered by the Committee at its fifty-fifth and fifty-seventh meetings (Annex 1, references *149* and *154*), and the tentative specifications were revised (Annex 1, reference *151*) and then superseded by full specifications (Annex 1, reference *156*). At its sixty-third meeting (Annex 1, reference *173*), the Committee evaluated biochemical data and the results of toxicological and human studies on *Tagetes* preparations with a high content of unesterified lutein (>80%) and established a group ADI of 0–2 mg/kg bw for lutein from *Tagetes erecta* and synthetic zeaxanthin.

At the present meeting, *Tagetes* extract was placed on the agenda following a request by CCFA to undertake a safety assessment and revision of specifications. However, the Committee noted that the information supplied by the sponsor referred to a substance with a higher content of carotenoid esters (>60%) compared with *Tagetes* extract; therefore, the Committee decided to name this extract "Lutein esters from *Tagetes erecta*". The INS number assigned to *Tagetes* extract could not be used as a synonym for this product.

Chemical and technical considerations

Lutein esters from *Tagetes erecta* is a dark yellow-brown solid insoluble in water and soluble in hexane. The product is obtained by solvent extraction of dried petals of *Tagetes erecta* L. (marigold), further purification and subsequent removal of solvents. Lutein esters account for the major part of the extract, and a smaller

proportion of zeaxanthin esters is also present, together with other carotenoids (xanthophylls), either free or as monoesters or diesters of fatty acids. The esters contain saturated long-chain fatty acids, such as myristic, palmitic and stearic acid, in various proportions, with palmitic acid being a major component. Waxes and fatty acid–containing moieties naturally occurring in the source material may also be present. Lutein, (all-*E*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol, is an oxygenated carotenoid often occurring with its isomeric xanthophyll zeaxanthin.

Biochemical data

The results of a number of studies conducted in experimental animals and humans have shown that the administration of lutein esters – in particular, lutein esters from *Tagetes erecta* – as dietary constituents or as nutritional supplements can lead to increases in levels of lutein in blood and accumulation of lutein in tissues (liver, adipose, eye). Lutein is also present in human milk. As assessed by increases in plasma levels of lutein, the bioavailability of lutein from the administration of lutein esters is equivalent to that from molar equivalent doses of lutein. Based on data obtained in studies with lutein, which generally report bioavailability in the range of 5–11%, the overall bioavailability of lutein from lutein esters is expected to be low, but may be higher with high fat intake.

Toxicological data

The toxicological study considered by the sixty-third meeting of the Committee (Annex 1, reference *173*) for the establishment of the ADI for lutein was a 13-week study in rats, from which the NOAEL was 200 mg/kg bw per day, the highest dose tested. In addition, results of in vitro and in vivo genotoxicity tests were negative, as were the results of a developmental toxicity study in rats at doses up to 1000 mg/kg bw per day, the highest dose tested.

The present Committee noted that the acute toxicity of lutein esters from *Tagetes erecta* in rats is low. No evidence of toxicity was apparent within 14 days of a single oral dose of lutein esters at 3750 mg/kg bw (equivalent to 2025 mg/kg bw expressed as lutein equivalents) or within 12 days following a single administration of a lutein ester preparation that provided up to 4000 mg/kg bw lutein equivalents.

In repeated-dose studies up to 90 days, no evidence of adverse effects was observed in rats at lutein ester doses up to 1000 mg/kg bw per day (equivalent to 540 mg/kg bw per day expressed as lutein equivalents), the highest dose tested. Long-term studies of toxicity in laboratory animals were not available.

The genotoxic potential of lutein esters from *Tagetes erecta* was evaluated in a bacterial reverse mutation assay, an in vitro mouse lymphoma assay and an

in vivo rat bone marrow micronucleus test. Lutein esters from *Tagetes erecta* were not mutagenic or genotoxic in any of these tests.

Reproductive toxicity studies in laboratory animals were unavailable. In a study of developmental toxicity with lutein esters in rats, there was no evidence of maternal or fetal toxicity at doses up to 540 mg/kg bw per day expressed as lutein equivalents (1000 mg/kg bw per day expressed as lutein esters), the highest dose tested.

Human studies

The available data evaluated by the Committee at its sixty-third meeting (Annex 1, reference *173*) indicated that dietary lutein is well tolerated in humans. After 20 weeks of mixed lutein ester supplementation at 15 mg/day, the effects in humans were limited to a reversible yellowish skin discoloration in some subjects.

The present Committee evaluated a number of studies in humans that have investigated the effects of the administration of lutein esters, primarily as nutritional supplements, on plasma levels of lutein, on the level of the xanthophyll-containing macular pigment and as a therapeutic agent for age-related macular degeneration. Although these studies in humans were not designed as part of the safety assessment process, it is noted that no adverse effects were reported in these published studies with doses of lutein esters from *Tagetes erecta* up to 30 mg/day for up to 140 days.

A clinical trial in preterm infants (<33 weeks of gestation) to assess potential retinal effects of daily lutein and zeaxanthin supplementation (0.14 mg + 0.0006 mg, respectively) for 36 weeks did not reveal any adverse effects.

Assessment of dietary exposure

Lutein esters from *Tagetes erecta* would be used in the same food categories and at the same use levels as those evaluated for lutein from *Tagetes erecta* at the sixty-third meeting of the Committee (Annex 1, reference *173*). That Committee estimated mean and 90th percentile dietary exposures for lutein plus zeaxanthin of approximately 7 and 13 mg/day, respectively (equivalent to 0.12 and 0.22 mg/kg bw per day, assuming a 60 kg body weight). Based on these results and assuming a conversion factor of 1.8 to reflect the increased molecular weight due to the fatty acid moieties, the present Committee estimated that exposure to lutein esters from *Tagetes erecta* would be up to 24 mg/day (equivalent to 0.4 mg/kg bw per day, assuming a 60 kg body weight). The use of lutein esters from *Tagetes erecta* is considered to be substitutional for the use of lutein from *Tagetes erecta* and therefore would not increase the dietary exposure to lutein.

At the sixty-third meeting, the Committee reported estimates for the dietary exposure to lutein from natural sources to be in the range of 1–6 mg/

day (approximately 0.01–0.1 mg/kg bw per day) based on a number of studies in North America and the United Kingdom. EFSA estimated that the mean dietary exposure to lutein from the diet due to its natural occurrence is up to 0.1 and 0.04 mg/kg bw per day for children and adults, respectively.

Evaluation

As the lutein (and zeaxanthin) esters from *Tagetes erecta* considered in the present evaluation undergo hydrolysis to lutein or zeaxanthin prior to systemic absorption, the biochemical and toxicological data on non-esterified lutein and zeaxanthin are relevant to the safety assessment of the lutein esters.

At the present meeting, the Committee concluded that there were sufficient toxicological data to complete a safety assessment of lutein esters from *Tagetes erecta*. The Committee considered the available toxicological data for lutein and lutein esters, together with the dietary exposure of the general population to lutein and lutein esters. New 13-week studies in rats with lutein esters resulted in a NOAEL of up to 540 mg/kg bw per day expressed as lutein equivalents, the highest dose tested. Additional information to support the safety assessment of lutein esters from *Tagetes erecta* includes the absence of any adverse effects in genotoxicity and developmental toxicity studies; and the absence of any reported adverse effects in humans administered lutein esters. These new data support the findings for other lutein preparations considered by previous Committees. No reproductive toxicity studies were available, but the Committee noted that the material is a common component of the diet, with no toxicity reported in available studies.

The Committee also noted that the human fetus is exposed to varying concentrations of lutein and zeaxanthin in utero, depending on maternal dietary exposure. Newborn infants continue to bioaccumulate lutein in many tissues, such as the retina, as a consequence of lutein being present in human milk.

The Committee concluded that there was no need to establish a numerical ADI. This decision was based on a number of factors, including the absence of any observed toxicity of lutein or lutein esters in any of the available toxicological studies in animals; the absence of any adverse effects in humans consuming lutein or lutein esters; the large MOE (>1500) between the NOAEL for lutein equivalents in a new 13-week study and the estimated dietary exposure of 0.32 mg/kg bw per day (from additive and natural sources); a 2-fold increase in the NOAEL for lutein as a result of another new 13-week study; and the fact that lutein esters from *Tagetes erecta* are considered to be substitutional for other lutein extracts.

At its sixty-third meeting, the Committee established a group ADI of 0–2 mg/kg bw for lutein from *Tagetes erecta* and synthetic zeaxanthin. At the

present meeting, the Committee established a temporary ADI "not specified" for lutein esters from *Tagetes erecta*. The ADI was made temporary because the specifications for lutein esters from *Tagetes erecta* were tentative.

The Committee considered establishing a group ADI "not specified" for lutein esters from *Tagetes erecta* that would include lutein from *Tagetes erecta* and synthetic zeaxanthin and related xanthophylls, but this would be possible only when the specifications for lutein esters from *Tagetes erecta* are finalized.

The Committee noted that limited information was received from the sponsor on the manufacturing process for and composition of the substance. Although a single compound was claimed as the major component of the extract, it was unclear whether this was the case, as it appeared that lutein and a number of similar carotenoids could be esterified with at least three different fatty acids. The analytical data from only one final product were supplied, and these did not give details on the composition of the carotenoids, the non-carotenoid portion of the extract, including waxes, and the fatty acid–containing fraction. The Committee prepared new specifications and, in view of the above limited information, made them tentative and requested the following information by the end of 2015 to complete the safety assessment:

- details on the manufacturing process, including purification steps;
- detailed analytical data on the full composition of at least five different batches of commercially available product to support the specifications;
- method of analysis to determine carotenoid composition; and
- method of analysis to determine the composition of the non-carotenoid lipidic fraction.

A toxicological monograph was prepared.

3.1.6 Octenyl succinic acid (OSA)–modified gum arabic

At its seventy-first meeting (Annex 1, reference *196*), the Committee evaluated the toxicological, chemical and technical data for OSA-modified gum arabic. In view of the similarities between OSA-modified gum arabic and the parent gum arabic, toxicological information for gum arabic was included in the toxicological monograph. At that meeting, the Committee decided to allocate a temporary ADI "not specified" to OSA-modified gum arabic, pending submission of data by the end of 2011 showing hydrolysis of OSA-modified gum arabic in the gastrointestinal tract to confirm the validity of using toxicological data on gum arabic in the evaluation of OSA-modified gum arabic. The Committee at its seventy-first meeting prepared new specifications for OSA-modified gum arabic.

At the seventy-fourth meeting (Annex 1, reference *205*), the Committee evaluated new data on the hydrolysis of OSA-modified gum arabic and reviewed the specifications. The Committee concluded that the results from the experiments on the hydrolysis of OSA-modified gum arabic did not unequivocally demonstrate that OSA-modified gum arabic hydrolyses completely in the stomach into gum arabic and OSA. Furthermore, the hydrolysis experiments showed inconsistencies with the reported stability of OSA-modified gum arabic in food. Therefore, the Committee deferred further evaluation of OSA-modified gum arabic and requested that the following data be provided by the end of 2013:

- data resolving the concern about the stability of OSA-modified gum arabic in food;
- data confirming that OSA-modified gum arabic is (completely) hydrolysed in the gastrointestinal tract, to confirm the validity of using gum arabic in the evaluation of OSA-modified gum arabic.

The temporary ADI was retained and the specifications were revised with changes in the test methods for the degree of esterification and for residual OSA content.

At the seventy-seventh meeting (Annex 1, reference *214*), the Committee evaluated a new study on the hydrolysis of OSA-modified gum arabic in simulated gastric fluid, simulated intestinal fluid and water. The Committee noted that complete hydrolysis of OSA-modified gum arabic under neutral pH conditions in simulated intestinal fluid or water, as reported in the study, was not in accordance with the claimed stability of the OSA ester linkage in aqueous solutions at the pH range of foods and beverages. The Committee considered that the spontaneous hydrolysis of OSA-modified gum arabic in water was unlikely to occur, which therefore raised doubts about the validity of the observed hydrolysis in the presence of gastrointestinal enzymes. In view of this, the Committee considered that the study did not unequivocally demonstrate that OSA-modified gum arabic hydrolyses completely in the stomach into gum arabic and OSA and that the validity of using toxicological data on gum arabic in the evaluation of OSA-modified gum arabic had not been confirmed.

The Committee also reviewed data on the stability of OSA-modified gum arabic in food and considered that these data demonstrated that OSA-modified gum arabic provided a stable emulsion in the two model food systems evaluated. However, the data did not unequivocally demonstrate that the OSA-modified gum arabic, at the molecular level, is stable (i.e. not hydrolysed) in food and beverages.

The Committee noted that ongoing studies on the stability of OSA-modified gum arabic in food may provide further information on its chemical fate in food and aqueous solutions, which could help to explain the contradictory

results of the hydrolysis study. Therefore, the Committee decided to retain the temporary ADI "not specified" pending submission of additional data on the stability of OSA-modified gum arabic in food, by the end of 2013.

The Committee also reviewed the specifications and noted that the purity test of degree of esterification in the current specifications should be replaced by the degree of substitution and requested information for an analytical method to measure the degree of substitution and results of the analysis of at least five commercially available batches. The specifications were made tentative pending submission of these data by the end of 2013.

At the present meeting, the Committee evaluated a demulsification study in simulated gastric fluid, simulated intestinal fluid and water using emulsions prepared with OSA-modified gum arabic. The Committee also evaluated data provided for the chemical characterization of OSA-modified gum arabic, as well as the method proposed to evaluate the degree of substitution and results of the analysis of commercial batches of the product in commerce.

Biochemical data

The demulsification study was performed in simulated gastric fluid, simulated intestinal fluid and water using emulsions prepared with OSA-modified gum arabic. The stability of the emulsions was verified using backscattering analysis, and undiluted emulsions were used as controls. Backscattering analysis conducted on undiluted emulsions indicated that the emulsions were stable over time, whereas the emulsions diluted 23 times in water, simulated intestinal fluid and simulated gastric fluid started to fail (demulsification) after approximately 3–4 hours, depending on the sample *(10)*. From backscattering analysis, it is possible to verify changes in particle size or concentration as a function of time. This technique provides information about the stability of an emulsion, but its use is not appropriate to study the dissociation of OSA from the gum at a molecular level.

Evaluation

The Committee is of the opinion that the demulsification study does not provide appropriate evidence that OSA-modified gum arabic is fully hydrolysed in the gastrointestinal tract to gum arabic and OSA and that the validity of using toxicological data on gum arabic in the evaluation of OSA-modified gum arabic has not been confirmed.

The Committee received data about the chemical composition of OSA-modified gum arabic in commerce, which were not in accordance with the existing specifications. The Committee noted that the residual (free) OSA was in the range of 3–4% (weight per weight [w/w]), whereas in the specifications monograph, a

value not higher than 0.3% is specified. The Committee was informed that there is no correlation between the free OSA content and emulsion stability and that the residual OSA does not contribute to the emulsifying properties, but is present as an impurity of the manufacturing process, which uses about 3% (w/w) OSA in the synthesis. The Committee noted that the high amount of free OSA in the product in commerce is not in accordance with the amount reported to be used in the synthesis of the manufacturing process. The submitted data did not clarify the nature of the linkage of the OSA and the gum; hydrogen bonding as well as covalent bonding (ester linkage) have been suggested by the sponsor. A ^{1}H nuclear magnetic resonance (NMR) method was proposed by the sponsor to determine the ratio of OSA to gum, but data on the degree of substitution using ^{1}H NMR or another appropriate method were not provided. Owing to the high amount of free OSA in the product, data obtained using ^{1}H NMR as well as ^{13}C NMR did not prove unequivocally that the gum was esterified.

The Committee revised the specifications and maintained the tentative status pending submission of data on the manufacturing process, including purification steps; chemical characterization of the product in commerce; updated analytical methods for the determination of esterified (bound) and residual (free) OSA; results of the analysis of at least five batches of product in commerce; and applicability of the high-performance liquid chromatographic method for the determination of residual OSA.

The Committee noted that in addition to the requested information, additional safety data may be needed to complete the evaluation of OSA-modified gum arabic. The Committee decided that the temporary ADI "not specified" will be withdrawn unless adequate data to complete the safety evaluation are submitted by the end of 2015.

No addendum to the toxicological monograph was prepared.

3.1.7 Octenyl succinic acid (OSA)–modified starch (starch sodium octenyl succinate)

Explanation

OSA-modified starch (starch sodium octenyl succinate) is listed in the GSFA *(3)* for use as a stabilizer, emulsifier and thickener in several food categories. The Committee previously reviewed OSA-modified starch at its thirteenth and twenty-sixth meetings (Annex 1, references *19* and *59*). At the twenty-sixth meeting, the Committee allocated an ADI "not specified" to OSA-modified starch because the only significant finding (corticomedullary calcium deposition in the kidney) was considered to be related to a marginal magnesium deficiency when carbohydrate comprises a major portion of the diet. The Committee prepared specifications for modified starches, including OSA-modified starch, at its thirty-

fifth meeting (Annex 1, reference *88*). These specifications were later revised by the Committee at the fifty-seventh, seventy-first and seventy-fourth meetings (Annex 1, references *154*, *196* and *205*).

At the request of CCFA at its Forty-fifth Session *(9)*, the Committee evaluated the safety of OSA-modified starch for use as an emulsifier in infant formula and in formula for special medical purposes intended for infants. Data submitted for evaluation included metabolic studies in rats, dogs and infants; short-term studies in rats, dogs and pigs; a long-term study in rats; and genotoxicity studies. Data from five trials in human infants were also included, as was some information from the post-marketing surveillance on a product containing OSA-modified starch.

Chemical and technical considerations

Starch sodium octenyl succinate (INS 1450; CAS No. 66829-29-6) is obtained by the modification of food starch with OSA. This modification involves controlled esterification by the introduction of lipophilic octenyl succinic groups from *n*-octenyl succinic anhydride to waxy starch pretreated with acid. The resulting *n*-octenyl succinate ester slurry undergoes several processing steps prior to being cooked under controlled temperature and pressure and spray-dried. The final starch sodium octenyl succinate product should contain not more than 3% octenyl succinyl groups and not more than 0.3% free OSA.

Biochemical data

The results of in vitro and in vivo studies conducted in experimental animals and humans demonstrate that OSA-modified starch is at least partially hydrolysed in the gastrointestinal tract by digestive enzymes to form OSA and native starch. The starch component undergoes typical carbohydrate digestion and absorption, whereas OSA is absorbed and excreted as the unchanged compound or metabolized via a combination of ω-, ω-1 and β-oxidation steps, similar to the metabolism of other branched-chain fatty acids, and then excreted. The fates of OSA-modified starch and the hydrolysed product OSA are similar in rats, dogs and human infants with respect to enzyme hydrolysis in the digestive tract, followed by absorption, metabolism and elimination, with the only difference being the amount of OSA excreted unchanged in the urine. The clinical data indicate that infants are also able to metabolize OSA to a number of different metabolites, including propane-1,2,3-tricarboxylic acid. While the degree of metabolism may differ among species, in general, the same metabolites are produced. Therefore, results from studies in rats and dogs conducted on OSA-modified starch are considered relevant for supporting the safety assessment of OSA-modified starch in humans.

Toxicological data

At its twenty-sixth meeting, the Committee noted that in a 90-day feeding study of OSA-modified starch at dietary levels up to 30% in the rat, the only significant finding was corticomedullary calcium deposition in the kidney. The twenty-sixth meeting of the Committee also made some general observations on the toxicological relevance of nephrocalcinosis in the rat. It noted that nephrocalcinosis may arise as a consequence of a physiological influence of carbohydrate intake on mineral metabolism, which needs to be taken into account in assessing the possible toxicological relevance of nephrocalcinosis. Since that time, there have been a large number of reports of nephrocalcinosis in response to exposures to carbohydrates with no changes in renal function, demonstrating that nephrocalcinosis in rats given large amounts of carbohydrates is not relevant to humans.

In a 90-day oral toxicity study not previously evaluated by the Committee, rats were fed diets containing 30% OSA-modified starch, equal to approximately 37 000 mg/kg bw per day. No test article–related adverse effects were reported.

The lack of toxicity of OSA-modified starch is further supported by the results of a long-term study in which no signs of toxicity were reported in rats fed OSA-modified starch at concentrations of up to 30% in the diet, equal to 17 and 21 g/kg bw per day for males and females, respectively, for up to 120 weeks.

OSA-modified starch has not shown evidence of genotoxicity in vitro. In addition, long-term dietary administration of OSA-modified starch produced no evidence of carcinogenicity in rats.

There were no data available on reproductive or developmental toxicity.

Special studies in young animals

Neonatal Beagle pups, 5–9 days old, were dosed twice daily by gavage with OSA-modified starch in water at doses of 5000 or 10 000 mg/kg bw per day for 6 weeks. Each dose group consisted of four puppies of each sex and was fed by a single dam. Body weight gain was lower than expected in all groups, including both water and starch controls, in the first 3 weeks, which was attributed to limited milk supply due to large litter size. Pups in the high-dose OSA-modified starch group were less active after 15 days and had decreased body weight gain compared with controls throughout the study. This effect could not be clearly attributed to OSA-modified starch because each treatment group of eight puppies was nursed by a single dam. No other toxicologically relevant findings were reported.

A follow-up study to investigate the reported effects in the high-dose group was conducted. Three-month-old Beagle pups were fed OSA-modified starch at dietary concentrations of 5.5%, 11.0% or 22.0%, equivalent to 3000, 6000 and 12 000 mg/kg bw per day, for 42 days. No clinical or histopathological

effects (including in the kidney) were reported for any dose group, but a decrease in body weight gain was reported for the high dose group animals compared with the starch-fed control animals. The differences in body weight gain were probably due to incomplete OSA-modified starch digestion, resulting in lower caloric intake.

In a GLP-compliant 3-week toxicity study conducted in neonatal piglets, OSA-modified starch was administered at doses of 1000, 2000 or 10 000 mg/kg bw per day via a feeding device 6 times per day. The piglets were administered OSA-modified starch for 3 weeks starting 2 days after birth, to model the 0- to 12-week period of development in human infants for which infant formula may be provided as the sole source of nutrition. OSA-modified starch was well tolerated in piglets and did not produce any definitive compound-related effects on growth or the clinical pathology parameters evaluated. No effects attributable to the test article were observed upon macroscopic or histopathological evaluation. As the digestive systems of neonatal swine and human infants are similar, the results of this study are relevant to the safety assessment of OSA-modified starch for use in infant formula.

Human studies

A single dose of 25 g of OSA-modified starch was well tolerated by fasting healthy non-diabetic adults and attenuated the post-prandial glycaemic response compared with glucose.

Two randomized, multicentre, double-blind clinical studies have been conducted to investigate the effects of infant formula supplemented with OSA-modified starch. In the first study, infants (approximately 50 per group) were fed either control formula or formula reported to contain OSA-modified starch as the sole source of nutrition at 1.33–1.47 g/100 mL beginning from 2–16 days of age for 120 days. No effects on growth were noted. There was no difference between the groups in illnesses or "symptoms of concern" in the infants as reported by the parents. In the second study, in which 168 infants 0–8 days of age were fed two similar casein hydrolysate formulas both containing less than 2% OSA-modified starch for comparison with commercially available control formula until day 28 of life, tolerance of the infants to OSA-modified starch formula was also examined. No issues with tolerability were reported with the OSA-modified starch formula.

In addition, two growth studies in which OSA-modified starch formula (concentration not specified) was included as the control formula have been evaluated. In these studies, 165 and 289 infants were fed OSA-modified starch containing formula from 14 through 120 days of age, and normal healthy growth and tolerance were reported. In a third growth study, 213 infants 0–9 days of age were fed infant formula containing OSA-modified starch at 16 g/L (1.6%),

calculated to give an OSA-modified starch exposure of approximately 2.5 g/kg bw per day through 112 days of age. Overall, the formula containing OSA-modified starch was well tolerated, and no health-related concerns were reported.

Post-marketing surveillance information on a recently globally marketed infant formula containing 2% OSA-modified starch to be used for special medical purposes has indicated that OSA-modified starch is well tolerated when administered to infants as recommended.

Assessment of dietary exposure

OSA-modified starch is proposed for use in infant formula and formula for special medical purposes intended for infants at levels up to 20 g/L formula.

Median infant formula consumption estimates were derived from the estimated energy requirements for fully formula-fed infants. It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. A further exposure scenario was considered using high (95th percentile) daily energy intakes reported for formula-fed infants. The highest reported 95th percentile energy intakes were for infants aged 14–27 days. For all dietary exposure estimates, a common energy density of formula of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy to the volume of formula ingested daily. The maximum proposed use level results in median estimated exposures to OSA-modified starch of up to 3.7 g/kg bw per day in infants aged 0–6 months, whereas infants with high (95th percentile) energy intakes may reach exposure levels of 4.4 g/kg bw per day.

Evaluation

At the twenty-sixth meeting, the Committee assigned an ADI "not specified" to OSA-modified starch. Since the time of that meeting, new data have become available, including a 90-day oral toxicity study, genotoxicity studies and a long-term toxicity and carcinogenicity study. All of the new data confirm the very low toxicity of OSA-modified starch, and the Committee confirmed the ADI "not specified".

Several new studies submitted were relevant to assessing the safety of OSA-modified starch in infant formula and formula for special medical purposes intended for infants.

Of the two studies conducted in neonatal animals, the study in piglets was considered the more relevant. The NOAEL of OSA-modified starch was 10 g/kg bw per day, the highest dose tested. The MOEs based on this NOAEL are 2.3 for the infants with the 95th percentile of energy intake (4.4 g OSA-modified starch/kg bw per day) and 2.7 at the median energy intake (3.7 g OSA-modified starch/kg bw per day).

In addition, several studies in human infants have shown that OSA-modified starch at concentrations up to 2% in infant formula is well tolerated; an exposure of 2.5 g/kg bw per day was provided for one of these studies. Post-marketing surveillance of an infant formula containing 2% OSA-modified starch also confirmed that it was well tolerated by infants.

The Committee took into account the overall low toxicity of OSA-modified starch, the conservatism in the NOAEL, which was the highest dose tested in a study in neonatal animals, and in the exposure assessments, as well as the supporting evidence from clinical trials and post-marketing surveillance and concluded that the consumption of OSA-modified starch in infant formula or formula for special medical purposes intended for infants is not of concern at use levels up to 20 g/L.

The Committee reviewed the existing specifications for OSA-modified starch. The Committee discussed limits on lead specifications for this and the other food additives for use in infant formula on the agenda, as described in section 2.4.1. The Committee revised the specifications monograph for modified starches, amending the analytical method for octenyl succinyl groups in starch sodium octenyl succinate in the monograph.

The Committee also discussed the fact that this food additive is part of the existing specifications monograph for modified starches along with 15 other modified starches. The Committee noted that it is difficult to revise individual specifications for any given modified starch within this specifications monograph. The Committee therefore recommended that the specifications monograph for the modified starches be split into 16 individual monographs.

A consolidated toxicological monograph was prepared.

3.1.8 Paprika extract

Explanation

At its fifty-fifth meeting in 2000 (Annex 1, reference *149*), the Committee concluded that paprika oleoresin is acceptable for use as a spice. This conclusion confirmed the outcome of an evaluation performed by the Committee at its fourteenth meeting in 1970 (Annex 1, reference *22*), which stated that the product was derived from a widely consumed natural foodstuff and there were no data indicative of a toxic hazard. The use of paprika oleoresin as a spice was considered to be self-limiting and obviated the need for an ADI. Paprika extract was placed on the agenda of the sixty-ninth meeting in 2008 (Annex 1, reference *190*) at the request of the Thirty-ninth Session of CCFA *(11)* for assessment of its safety as a food colour, specifications and dietary exposure. CCFA asked if the existing safety assessment and specifications for paprika oleoresin for use as a spice could be extended to the use as a food colour. As the source material and

the manufacturing process differ for paprika preparations used as a spice and as a food colour, the name "paprika extract" was adopted for use as a food colour.

At the sixty-ninth meeting, the Committee evaluated the use of paprika extract as a food colour. A toxicological monograph and new specifications were prepared. However, the safety assessment was not completed and the new specifications were made tentative pending the receipt of additional information on paprika extract. The Committee requested information on the concentrations of capsaicin present in the extracts and additional information about the composition of batches of extract produced by a variety of manufacturers. The Committee also wanted assurance that the material tested in the 90-day and long-term studies in rats was representative of all commercial production of paprika extract. At the seventy-seventh meeting of the Committee (Annex 1, reference *214*), following receipt of additional analytical data, the specifications were revised, and the tentative status was removed. The Chemical and Technical Assessment prepared at the sixty-ninth meeting was also revised to include the composition of commercial preparations.

At the present meeting, the Committee reconsidered the toxicological data and the dietary exposure in order to establish an ADI for paprika extract for use as a food colour. These data are presented in detail in the toxicological monograph prepared at the sixty-ninth meeting and are summarized only briefly here. No new toxicity data or data on dietary exposure have been submitted to the Committee since its sixty-ninth meeting.

Chemical and technical considerations

Information on the composition of six extracts selected from different producers of commercial paprika extracts was submitted by the sponsor for the seventy-seventh meeting of the Committee in 2013 (Annex 1, reference *214*). These samples originated from India (one), Spain (four) and Peru (one). The content of total carotenoids in these extracts of paprika varied between 4.2% and 8.4%. The major carotenoid in these extracts was capsanthin, which constituted between 36.7% and 56.1% of total carotenoids. Other major carotenoid pigments were (13*Z*)-β-carotene (6.6–10.7%), (15*Z*)-β-carotene (4.2–11.9%) and zeaxanthin (3.0–12.4%). There were smaller amounts of other carotenoids, including capsorubin, which constituted between 0.6% and 2.3% of total carotenoids. Together, these are referred to as total carotenoids present in paprika extract. Levels of capsaicin were below the limit of detection (30 mg/kg) in five of the six samples and were present in the sixth sample at less than 100 mg/kg. Levels of all capsaicinoids were below 200 mg/kg (0.02% of the substance). At the seventy-seventh meeting, the Committee set the maximum content of capsaicinoids at 200 mg/kg and the maximum content of arsenic at 1 mg/kg. In addition to the

principal colour components, paprika extract also contains neutral lipids and phospholipids composed of a variety of saturated and unsaturated fatty acids. The additional data did not reveal the presence of impurities (including heavy metals and solvents) that would raise concerns for consumer safety. The composition of the paprika extract tested in the 90-day and long-term toxicity studies in rats was comparable to the composition of the extracts used in commerce as a food colour.

Biochemical data

The systemic bioavailability of carotenoids present in paprika preparations was investigated in two human studies. In one study, capsanthin, the major carotenoid present in a paprika oleoresin administered orally as a single dose to nine volunteers, was not present in the chylomicron fraction of whole blood between 1 and 12 hours after dosing. Other carotenoids (zeaxanthin, β-carotene and β-cryptoxanthin) reached a maximum concentration in blood at 6 hours *(12)*. In another study, 8 hours after four male volunteers ingested paprika juice (single dose equivalent to 34.2 μmol capsanthin), capsanthin was detected in the plasma at 0.1–0.29 μmol/L. After the volunteers ingested paprika juice 3 times daily for 1 week (daily dose equivalent to 16.2 μmol capsanthin), plasma concentrations of capsanthin reached a plateau of 0.1–0.12 μmol/L in 2–7 days *(13)*. The results indicate that the systemic bioavailability of capsanthin is considered to be low.

Toxicological data

In a 13-week study, F344 rats were given paprika extract with a carotenoid content of 7.5% and a capsaicin content of less than 0.01% *(14)*. The paprika extract was administered at dietary levels of 0% (basal diet), 0.62%, 1.25%, 2.5% and 5%. No significant adverse effects were observed at any dose level. The NOAEL was 5% in the diet, the highest concentration tested, equal to 2950 and 3200 mg/kg bw per day for males and females, respectively.

In a combined 52-week toxicity and 104-week carcinogenicity study, rats were given diets containing 0%, 2.5% or 5% paprika extract (a carotenoid content of 7.5% and a capsaicin content of <0.01%, as in the 13-week study described above). All animals were subjected to a complete pathological evaluation. No evidence of adverse effects, including carcinogenicity, was observed at either dose level or time point. The NOAEL was considered to be 5% in the diet, equal to 2388 and 2826 mg/kg bw per day (52 weeks) and 2052 and 2324 mg/kg bw per day (104 weeks) for males and females, respectively *(15)*.

The Committee noted that mutagenicity/genotoxicity tests conducted on extracts of chili peppers and samples of capsaicin have yielded mixed, inconsistent and often contradictory results. However, results of more recent studies with

pure capsaicin support the conclusion that it is not genotoxic in standard in vitro and in vivo assays. Furthermore, Hallagan and co-workers *(16)* concluded that a weight-of-evidence analysis indicates that paprika is not genotoxic, which is in agreement with the negative results of the 104-week carcinogenicity study in rats.

The Committee noted that reproductive and developmental toxicity studies for paprika extract were not available.

Assessment of dietary exposure

There are limited data on the potential dietary exposure to total carotenoids from use of paprika extract as a food colour. Dietary exposures to total carotenoids from paprika extract were based on consumption data from the United Kingdom and France. Assuming that 7% of paprika extract was total carotenoids, the estimated mean dietary exposure to total carotenoids for the French population was 2.3 mg/day, and the estimated dietary exposure for a high consumer was 7.0 mg/day. For the United Kingdom, survey data (based on four age categories) yielded mean estimated dietary exposures to total carotenoids from paprika extract of 2.9–6.9 mg/day. The exposure at the 95th percentile was estimated to be 6.3–13.2 mg/day. Exposure to capsaicin and all capsaicinoids from paprika extract is considered to be less than 0.05 mg/day, based on the low levels of all capsaicinoids present in paprika extract used as a food colour.

Evaluation

The results of a 90-day dietary toxicity study and a combined 52-week toxicity and 104-week carcinogenicity study in rats, both conducted with a paprika extract representative of the various extracts used in commerce as a food colour, did not provide evidence of any adverse effects. The NOAEL for paprika extract in both studies was 5% in the diet, the highest concentration tested, equal to 2052 mg/kg bw per day (calculated by the authors for the 104-week carcinogenicity study).

The Committee noted that the weight of evidence obtained in genotoxicity studies indicates that paprika extract is not genotoxic.

Although reproductive and developmental toxicity studies for paprika extract were not available, the Committee noted that the major components of paprika extracts were mixtures of neutral lipids and phospholipids containing saturated and unsaturated fatty acids, which are unlikely to present a reproductive or developmental hazard. Also, the Committee was aware of the results of a GLP-compliant developmental toxicity study in rats that had been performed with lutein (administered at 10% in the diet), a carotenoid of similar structure to capsanthin *(17)*. No developmental abnormalities were observed in the study with lutein at any dose level, and a NOAEL of 1000 mg/kg bw per day, the highest dose tested, was identified. Thus, the Committee concluded that paprika

extract meeting the specifications for use as a food colour is unlikely to pose a reproductive or developmental hazard.

The total carotenoid level in the paprika extracts used in the toxicity studies described above was 7.5%, which is in the mid-range of the levels in commercial paprika extracts used as colours. The NOAEL of 2052 mg/kg bw per day is equivalent to 153 mg/kg bw per day for paprika extracts when expressed as total carotenoids. The Committee applied an uncertainty factor of 100 to the NOAEL of 153 mg/kg bw per day and established an ADI for paprika extract used as a food colour of 0–1.5[4] mg/kg bw, expressed as total carotenoids.

The assessment of dietary exposure to paprika extract used as a colour was based on exposure to total carotenoids in paprika extract. Based on survey data, the highest exposure at the 95th percentile was estimated to be 6.3–13.2 mg/day (equivalent to 0.1–0.2 mg/kg bw per day, based on a body weight of 60 kg), which is below the ADI. The Committee concluded that dietary exposure to paprika extract used as a food colour does not present a health concern.

No addendum to the toxicological monograph was prepared, and the specifications were maintained.

3.1.9 Pectin

Explanation

Pectins (INS 440; CAS No. 9000-69-5) are used as a gelling, thickening and stabilizing agent and are approved for use in general foods all over the world. Pectins as food additives have been evaluated by the Committee at its thirteenth, seventeenth, eighteenth, nineteenth and twenty-fifth meetings (Annex 1, references *19*, *32*, *35*, *38* and *56*). At its twenty-fifth meeting in 1981 (Annex 1, reference *56*), the Committee established a group ADI "not specified" for pectin and amidated pectin.

Current specifications for pectins were established by the Committee at its seventy-first meeting (Annex 1, reference *198*), superseding the previous specifications set by the Committee at its sixty-eighth meeting (Annex 1, reference *189*).

At the present meeting, the Committee was asked to consider the safety of using non-amidated pectin in infant formula and formula for special medical purposes intended for infants. The Committee reviewed new data published since the twenty-fifth meeting, in particular data of relevance to the products being considered. The data submitted for evaluation included studies using pectin: a biotransformation study in rats, a study in neonatal pigs and a human infant study. In addition, a number of studies used pectin-derived oligosaccharides,

[4] The Committee noted that although derived values, such as health-based guidance values, should be rounded to a single significant figure, it decided to use two significant figures in the present case, as the impact of rounding to one significant figure would be more than 30%.

which the sponsors considered relevant to the evaluation of pectin. These studies were short-term toxicity studies in rats, a reproductive toxicity study in rats, genotoxicity studies and four studies in human infants.

Chemical and technical considerations

Pectin is a complex heteropolysaccharide that consists mainly of the partial methyl esters of polygalacturonic acid and their sodium, potassium, calcium and ammonium salts. It is obtained by aqueous extraction of appropriate edible plant material, usually citrus fruits or apples. The average molecular weight of food-grade pectin will vary depending upon the pectin source and processing and is expected to range from 100 to 200 kDa. Amidated pectin is prepared by treatment of pectin with ammonia under alkaline conditions. The existing specifications for pectins cover both pectin and amidated pectin.

Pectin is used in infant formula as a thickener to increase the viscosity of the formula and as a stabilizer to maintain the homogeneity of the formula throughout its shelf life. According to the sponsor, amidated pectin is not used in infant formula.

The Committee was made aware that a further pectin product is available on the market. This product, known as pectin-derived acidic oligosaccharides (pAOS), is produced by enzymatic hydrolysis of pectin. pAOS has not been evaluated by the Committee and is not covered by the existing specifications for pectins.

Biochemical data

Pectin is a non-digestible carbohydrate that is extensively fermented by the microflora in the gastrointestinal tract to oligogalacturonic acids, which are then further metabolized to short-chain fatty acids, such as acetate, propionate and butyrate.

pAOS is a product of the digestion of food-grade pectin and consists of small polymers predominantly of molecular weight of no more than 3800 Da. Manufactured pAOS is similar to products formed from pectin in the gastrointestinal tract. The Committee agreed that data from studies on pectin-derived oligosaccharides can support conclusions reached on the basis of data from studies that have tested pectin.

Toxicological data

The toxicity of pAOS was studied in a one-generation study in rats in which there was a 13-week investigation of F_1 offspring. Male and female parental (F_0) animals were fed test and control diets starting 4 weeks prior to mating and throughout the mating, gestation and lactation periods until weaning of the

offspring (F_1 rats). There were four diets used in the study. Two were control diets, one being the standard rodent diet plus 10% potato starch and the other a reference control diet that was the standard rodent diet plus 10% short-chain fructo-oligosaccharides, which mimic the neutral oligosaccharides of human milk. The test diets contained 5% and 10% pAOS. The calculated intakes of pAOS in the F_0 females during the premating and gestation periods were 3.1 and 6.2 g/kg bw per day in the 5% and 10% pAOS groups, respectively. The overall intake of pAOS during the early phases of the testing up to the end of lactation was up to 7.1 g/kg bw per day.

No treatment-related effects were seen in clinical signs, body weights, growth rate, feed intake or reproductive indices in the dams. Pups were also unaffected in their general condition or on histopathological examination. In the subsequent subchronic phase of the study with rats selected from the F_1 generation, effects attributable to the test substance were increased urinary sodium concentration and excretion and urinary pH in males in both dose groups and increased urinary sodium excretion and urinary pH in females in the 10% group. The study authors attributed the increased sodium excretion to high sodium levels (3120 mg/100 g) in pAOS. Caecum weights were increased in the 5% and 10% dose groups, and kidney weights were also increased in males at the 10% dose. Taking into account that caecum weight increases are commonly observed with high-fibre diets in rats and that there was no indication of disturbed renal function or kidney histopathology, the Committee concluded that these effects were of no toxicological relevance. In addition, an increase in diffuse hyperplasia of the bladder epithelium was seen in males and females of the high-dose group, with the effect reaching statistical significance only in males.

A 13-week study in rats was carried out to investigate the cause of the effects seen in the above study and to determine a NOAEL. The dose groups were 1%, 2.5% and 10% pAOS; in addition, there was an additional group fed test material containing 10% pAOS and ammonium chloride to test whether acidification of the urine could prevent the diffuse hyperplasia of the bladder epithelium. The overall intakes of pAOS, with and without ammonium chloride, in high-dose rats were 7.2 and 7.1 g/kg bw per day, respectively. The overall intakes of pAOS in the 1% and 2.5% dose groups were 0.7 and 1.7 g/kg bw per day, respectively. Diffuse hyperplasia of the bladder epithelium was again seen in males of the 10% pAOS group, and a similar change, albeit less prominent, was observed in females of this group. Very slight diffuse hyperplasia of the bladder epithelium was also noted in one female of the 1% pAOS group, but not at the next higher dose level, and this finding was therefore considered incidental. Urinary sodium excretion was increased in rats fed 10% pAOS, with and without ammonium chloride, and the urinary sodium concentration was higher in males than in females. The incidence and severity of the diffuse hyperplasia of the bladder epithelium

in this study were lower than in the first study. Rats fed pAOS in combination with ammonium chloride had lower urinary pH, and diffuse hyperplasia of the bladder epithelium was not observed. The authors commented that this finding showed that the hyperplasia was caused by the concurrent increase in urinary sodium concentration and pH, a condition known to predispose rats to hyperplasia of the bladder epithelium, and not to pAOS itself. Diffuse hyperplasia of the bladder epithelium resulting from a concurrent increase in urinary pH and urinary sodium ion concentration is a well known phenomenon in rats and not frequently observed in other animal species. On this basis, the Committee agreed that because of the species specificity of the response, diffuse hyperplasia of the bladder epithelium induced by this mechanism in rats is considered not to be of relevance to humans; the Committee concluded that the NOAEL of pAOS was about 7 g/kg bw per day, the highest dose tested.

There were no new data available on long-term toxicity or carcinogenicity.

The genotoxicity of pAOS was examined in a bacterial reverse mutation assay, a mouse lymphoma assay, a chromosome aberration study in Chinese hamster ovary cells and a rat micronucleus test. The bacterial reverse mutation assay with and without metabolic activation was negative. Positive responses were seen in the other two in vitro studies only in the absence of metabolic activation and in the presence of significant cytotoxicity. The mouse lymphoma assay showed a positive response at 2920 μg/mL and an equivocal response at 3590 μg/mL (the highest concentration tested). Cytotoxicity was observed at these concentrations, and the results were determined to be equivocal. The chromosome aberration study indicated that the positive response and cytotoxicity were related to the solvent (dimethyl sulfoxide), and the authors concluded that pAOS was cytotoxic, but not clastogenic. No increases in micronuclei were observed in bone marrow polychromatic erythrocytes of rats fed pAOS at approximately 7 g/kg bw per day in the one-generation study described above. There was no change in the ratio of normochromatic to polychromatic erythrocytes. Overall, the Committee concluded that pAOS is not genotoxic.

In the one-generation study in rats dosed with pAOS, there was no effect seen on reproduction or on general condition of the pups, litter size, pup viability or sex ratio.

Special studies in young animals

Neonatal piglets were administered pectin in milk replacer formula as their sole source of nutrition for 3 weeks after birth at a concentration of 0.5, 3.0 or 10.0 g/L (doses calculated to be 142, 847 and 3013 mg/kg bw per day for males and 141, 879 and 3094 mg/kg bw per day for females, respectively). The milk replacer formula was offered to the piglets 6 times per day at a dose volume of 500 mL/kg

bw, and the intake of pectin was determined from the amount of milk replacer formula consumed per day. Decreased intake of the milk replacer was correlated with mean decreased body weights in males in the 10.0 g/L dose group (3.88 kg versus 4.35 kg in controls) from day 13, which reached statistical significance (5.22 kg versus 6.47 kg in controls) at termination (day 21). For females, the mean body weights were consistently lower in the 10.0 g/L dose group from day 15 (4.3 kg versus 4.5 kg in controls), and at termination they were 5.65 kg compared with 5.95 kg in controls. Feed consumption in males in the 10.0 g/L dose group was decreased by 30% by the end of the study. The body weights in the two lowest dose groups were similar to those of controls. There were minor changes seen in haematology tests, but they were not considered to be toxicologically relevant. Caecum and colon weights were statistically significantly increased in the top two dose groups.

No treatment-related histopathological changes were seen in the intestinal tract. This study showed that pectin in doses up to 847 mg/kg bw per day in milk replacer administered to neonatal pigs over a 3-week period was well tolerated. A decreased intake of the milk replacer and an associated decrease in body weight were seen at a pectin dose of 3013 mg/kg bw per day.

Human studies

One study has investigated the effects of pectin in preterm human infants. Infants (n = 74) receiving human milk fortified with a liquid infant milk fortifier (stated by the sponsor to contain 0.085% pectin after addition to the human milk) for 28 days were compared with infants (n = 72) receiving human milk fortified with a control milk fortifier. At the conclusion of the study, the pectin-fed infants had a significantly higher linear growth rate, along with greater increases in weight, length and head circumference. No treatment-related adverse events were observed. These results suggest that pectin at 0.085% in infant formula was well tolerated in preterm infants.

Four studies investigated the effects of infant formula containing pAOS on term human infants. In one study, a cohort of healthy infants (n = 414) enrolled in the study at ages 20–42 days was fed an infant formula containing 1.2 g/L (0.12%) pAOS until the age of 12 months. No differences were reported in growth, gastrointestinal tolerance or stool frequency between infants fed the pAOS formula and infants fed a control formula, but stool consistency was softer in infants fed the pAOS formula. A second study showed that pAOS did not affect growth characteristics in healthy infants (n = 27) when they consumed a formula containing 0.2% pAOS daily (240 mg/kg bw per day) for 2 months.

In the last two studies, the effect of pAOS on gut microflora in infants was investigated. In one study, term infants (n = 16) were given an infant formula

containing 0.2% pAOS every day for 6 weeks. Microbiological analysis of faecal samples from the group fed pAOS infant formula indicated no effect on the counts of *Bifidobacterium* sp., *Clostridium* sp., *Escherichia coli* or *Enterobacter* sp. In the final study, in which term infants (n = 82) were fed an infant formula containing 0.2% pAOS (equivalent to 240 mg/kg bw per day), there was no effect on the same bacteria as in the previous study compared with those receiving a control formula.

Assessment of dietary exposure

The maximum proposed use level of non-amidated pectin in formula intended for infants aged 0–12 weeks is 5 g/L.

Median infant formula consumption estimates were derived from estimated energy requirements for fully formula-fed infants. It should be noted that the energy requirements of formula-fed infants are greater than those of breastfed infants, although this disparity decreases with increasing age. A further exposure scenario was considered, using high (95th percentile) daily energy intakes reported for formula-fed infants. The highest reported 95th percentile energy intakes were for infants aged 14–27 days. For all dietary exposure estimates, a common energy density of formula of 67 kcal/100 mL (280 kJ/100 mL) was used to convert energy to the volume of formula ingested daily.

The proposed use level for pectin in infant formula results in median estimated exposures to pectin of 0.75–0.91 g/kg bw per day in infants ages 0–12 weeks, whereas infants with high (95th percentile) energy intakes may reach an exposure level of 1.1 g/kg bw per day.

Evaluation

At the twenty-fifth meeting, the Committee assigned an ADI "not specified" to pectin and amidated pectin. At the present meeting, the Committee reviewed new data that were particularly of relevance to the safety assessment of the use of non-amidated pectin in infant formula and formula for special medical purposes intended for infants. Many of the data reviewed by the Committee were from studies that had examined pectin-derived oligosaccharides such as pAOS. As manufactured pAOS is similar to products formed from pectin in the gastrointestinal tract, the Committee concluded that these studies were relevant to the evaluation of pectin in infant formula.

In short-term toxicity studies, feeding test material containing pAOS to rats for 13 weeks was reported to cause diffuse hyperplasia of the bladder epithelium, which was considered to be species specific and not relevant to humans. The NOAEL of pAOS in these studies was about 7 g/kg bw per day, the highest dose tested.

Genotoxicity studies on pAOS gave negative or equivocal results in vitro and a negative result in vivo following 90 days of administration in the rat at doses up to about 7 g/kg bw per day. The Committee concluded that pAOS is not genotoxic.

In a 3-week study in neonatal pigs fed pectin-containing milk replacer, the NOAEL of pectin was 847 mg/kg bw per day, with decreased feed intake and body weight gain observed at 3013 mg/kg bw per day. The Committee concluded that the neonatal pig is an appropriate model for the human infant. Using the NOAEL from this study, the MOEs were estimated to be 0.9 for infants with median energy intake and 0.8 for infants with high (95th percentile) energy intake.

In human infant studies, one showed that pectin in infant formula was well tolerated by preterm infants at a concentration of 0.085%. Four studies with pAOS (at up to 0.2%) in formulas provided some support for the tolerance of infants to pectin.

The Committee concluded that estimated exposure to pectin from its use in infant formula is in the region of the NOAEL of pectin derived from the neonatal pig study (847 mg/kg bw per day) and close to the LOAEL based on decreased feed intake and body weight gain. Although no overt toxicological effects were observed in the neonatal pigs, decreased food intake and body weight gain would be considered an undesirable effect in human infants. The available clinical studies were mainly conducted with pectin or pectin-derived oligosaccharides at concentrations of 0.2% or less and therefore do not provide support for tolerance and normal growth at the maximum proposed use level. Therefore, the Committee concluded that the use of pectin in infant formulas at the maximum proposed use level (0.5%) is of concern.

The Committee requests additional data to support the safety evaluation of pectin in infant formula, including an explanation for the decreased feed intake and body weight gain in neonatal pigs.

The Committee at its seventy-first meeting (2009) had prepared specifications for pectins. The Committee discussed limits on lead specifications for this and the other food additives for use in infant formula that were on the agenda, as described in section 2.4.1. At the present meeting, the specifications for pectins were maintained.

An addendum to the toxicological monograph was prepared.

3.2 Revision of specifications

3.2.1 Citric acid

Citric acid was on the agenda at the present meeting at the request of the Forty-fifth Session of CCFA *(9)* due to an inconsistency in the limit test for oxalate in its specifications monograph. The current provisions of the oxalate limit test include a maximum value for absorbance that does not represent the limit specified for oxalic acid. The test method in the Compendium of Food Additive Specifications, FAO JECFA Monographs 1, Volume 4 (Annex 1, reference *180*), was revised (see section 2.4.3). The revised method will be published in the Compendium of Food Additive Specifications, FAO JECFA Monographs 16 (2014).

The specifications for citric acid were revised with a reference to the revised method.

3.2.2 Gellan gum

Gellan gum was placed on the agenda of the present meeting at the request of the Forty-fifth Session of CCFA *(9)* to consider the use of ethanol in the manufacturing process as an alternative to 2-propanol. The specifications were revised accordingly, and a revised method for the determination of residual solvents using headspace gas chromatography was included. The assay method in the specifications refers to the alginates assay method in Volume 4 of the Combined Compendium of Food Additive Specifications (Annex 1, reference *180*), which was replaced with a new method without the use of mercury (see section 2.4.2).

3.2.3 Polyoxyethylene (20) sorbitan monostearate

The Committee at its twenty-fifth meeting in 1981 (Annex 1, reference *56*) had prepared specifications for polyoxyethylene (20) sorbitan monostearate. At its Forty-fifth Session *(9)*, CCFA requested a revision of the specifications, in particular a change in the saponification and hydroxyl values. Based on the analytical data provided, the Committee revised the specifications to incorporate the proposed changes.

3.2.4 Potassium aluminium silicate

PAS was on the agenda of the present meeting at the request of the Forty-sixth Session of CCFA *(2)* for consideration of deleting the functional use of carrier in the specifications monograph for PAS. According to Codex *(18)*, a carrier is "a food additive used to dissolve, dilute, disperse or otherwise physically modify a food additive or nutrient without altering its function (and without exerting any technological effect itself) in order to facilitate its handling, application or

use of the food additive or nutrient". At its seventy-seventh meeting (Annex 1, reference *214*), the Committee revised the specifications for PAS and included "carrier (used as a carrier substrate in pearlescent pigments made with titanium dioxide and/or iron oxide)" as one of its functional uses. At the present meeting, the Committee reviewed the existing data as well as new information received from the sponsor and noted that PAS stabilizes the formed layers of titanium dioxide and/or iron oxide in the PAS-based pearlescent pigments. Therefore, the Committee concluded that PAS exerts a technological effect in the PAS-based pearlescent pigments; as a result, PAS could not be considered to function as a carrier according to the Codex definition. Hence, the Committee decided to delete the functional use as carrier in the specifications monograph for PAS.

The specifications were revised accordingly.

3.2.5 *Quillaia* extract (Type 2)

The Committee at its sixty-fifth meeting in 2005 (Annex 1, reference *178*) had prepared specifications for *Quillaia* extract (Type 2). At its Forty-fifth Session *(9)*, CCFA requested a revision of the specifications concerning the upper limit in the loss on drying specification, from 80% to 90%. Based on the analytical data submitted, the Committee revised the specifications to incorporate the proposed change.

4. Flavouring agents

4.1 Flavouring agents evaluated by the Procedure for the Safety Evaluation of Flavouring Agents

Assignment to structural class

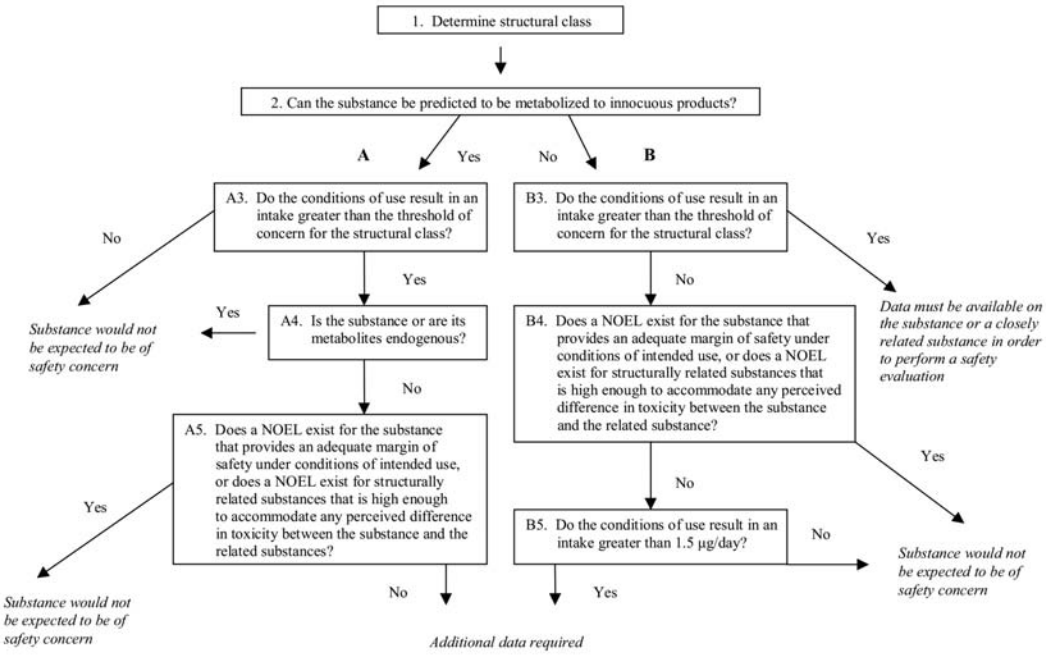
Eight groups of flavouring agents were evaluated using the Procedure for the Safety Evaluation of Flavouring Agents, as outlined in Fig. 1 (Annex 1, references *116*, *122*, *131*, *137*, *143*, *149*, *154*, *160*, *166*, *173* and *178*). In applying the Procedure, the chemical is first assigned to a structural class as identified by the Committee at its forty-sixth meeting (Annex 1, reference *122*). The structural classes are as follows:

- *Class I.* Flavouring agents that have simple chemical structures and efficient modes of metabolism that would suggest a low order of toxicity by the oral route.
- *Class II.* Flavouring agents that have structural features that are less innocuous than those of substances in class I but are not suggestive of toxicity. Substances in this class may contain reactive functional groups.
- *Class III.* Flavouring agents that have structural features that permit no strong initial presumption of safety or may even suggest significant toxicity.

A key element of the Procedure involves determining whether a flavouring agent and the product(s) of its metabolism are innocuous and/or endogenous substances. For the purpose of the evaluations, the Committee used the following definitions, adapted from the report of its forty-sixth meeting (Annex 1, reference *122*):

- *Innocuous metabolic products* are defined as products that are known or readily predicted to be harmless to humans at the estimated dietary exposure to the flavouring agent.
- *Endogenous substances* are intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included. The estimated dietary exposure to a flavouring agent that is, or is metabolized to, an endogenous substance should be judged not to give rise to perturbations outside the physiological range.

Figure 1
Procedure for the Safety Evaluation of Flavouring Agents



Assessment of dietary exposure

Maximized survey-derived intake (MSDI)

Estimates of the dietary exposure to flavouring agents by populations are based on annual volumes of production. These data were derived from surveys in Europe, Japan and the United States of America (USA). Manufacturers were requested to exclude use of flavouring agents in pharmaceutical, tobacco or cosmetic products when compiling these data. When using these production volumes to estimate dietary exposures, a correction factor of 0.8 is applied to account for under-reporting.

$$\text{MSDI (µg/day)} = \frac{\text{annual volume of production (kg)} \times 10^9 \text{ (µg/kg)}}{\text{population of consumers} \times 0.8 \times 365 \text{ days}}$$

The population of consumers was assumed to be 41×10^6 in Europe, 13×10^6 in Japan and 31×10^6 in the USA.[5]

[5] Population counts in 2010 were reported by IOFI *(19)* to be 410 million for Europe (EU-16 plus Turkey and Switzerland), 309 million for the USA and 128 million for Japan.

Single-portion exposure technique (SPET)

The SPET was developed by the Committee at its sixty-seventh meeting (Annex 1, reference *184*) to account for presumed patterns of consumer behaviour with respect to food consumption and the possible uneven distribution of dietary exposures among consumers of foods containing flavouring agents. It is based on reported use levels supplied by the industry. This single portion–derived estimate was designed to account for individuals' brand loyalty to food products and for niche products that would be expected to be consumed by only a small proportion of the population. Its use in the Procedure was endorsed at the sixty-ninth meeting of the Committee (Annex 1, reference *190*) to render the safety assessment more robust, replacing the sole use of MSDI estimates with the higher of the highest MSDI or the SPET estimate as the exposure estimate in the decision-tree. The Committee also agreed that it would not be necessary to re-evaluate flavouring agents that had already been assessed previously using the Procedure.

The SPET provides an estimate of dietary exposure for an individual who consumes a specific food product containing the flavouring agent every day. The SPET combines an average (or usual) added use level provided by the flavour industry with a standard portion size from 75 predefined food categories as described by the Committee at its sixty-seventh meeting. The standard portion is taken to represent the mean food consumption for consumers of these food categories. Among all the food categories with a reported use level, the calculated dietary exposure from the single food category leading to the highest dietary exposure from one portion is taken as the SPET estimate:

$$\text{SPET (}\mu\text{g/day)} = \text{standard portion size of food category } i \text{ (g/day)} \times \text{use level for food category } i \text{ (}\mu\text{g/g)}$$

The highest result is used in the evaluation.

The use level data provided by industry for each flavouring agent evaluated at this meeting and used in the SPET calculations are available on the WHO JECFA website at http://www.who.int/foodsafety/publications/jecfa/en/.

Consideration of combined intakes from use as flavouring agents

The safety assessment of possible combined intakes of flavouring agents was based on the presence of common metabolites or a homologous series (as proposed at the sixty-eighth meeting; Annex 1, reference *187*) and using the MSDI exposure assessment (as proposed at the sixty-ninth meeting; Annex 1, reference *190*).

4.1.1 Aliphatic and alicyclic hydrocarbons

Introduction

The Committee evaluated six additional flavouring agents belonging to the group of aliphatic and alicyclic hydrocarbons. The additional flavouring agents included five aliphatic alkenes (Nos 2191, 2192, 2194, 2195 and 2196) and one alicyclic hydrocarbon (No. 2197). The Committee decided not to evaluate α-ionene (No. 2193), which was also submitted for evaluation as part of this group, because it determined that the chemical structure of this aromatic hydrocarbon does not fit into the aliphatic and alicyclic hydrocarbons group. The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents. None of these flavouring agents has previously been evaluated by the Committee.

The Committee previously evaluated 20 other members of this group of flavouring agents at its sixty-third meeting (Annex 1, reference *173*). The Committee concluded that all 20 flavouring agents were of no safety concern at estimated dietary exposures. One member of this group, *d*-limonene (No. 1326), was previously evaluated by the Committee at its thirty-ninth meeting (Annex 1, reference *101*) and was assigned an ADI of 0–1.5 mg/kg bw. At its forty-first meeting (Annex 1, reference *107*), the Committee re-evaluated the ADI for *d*-limonene and recommended that it be withdrawn and replaced with an ADI "not specified". At the sixty-third meeting, the ADI "not specified" was maintained for *d*-limonene (Annex 1, reference *173*).

Three of the six flavouring agents in this group (Nos 2191, 2192 and 2195) have been reported to occur naturally in foods. They have been detected in, for example, apples, citrus fruits, mushrooms, peanuts, walnuts, cheese, eggs, milk, honey, beef, pork and chicken.

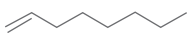
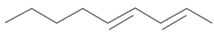
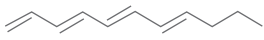
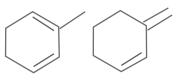
Assessment of dietary exposure

The total annual volume of production of the six flavouring agents belonging to the group of aliphatic and alicyclic hydrocarbons is approximately 0.2 kg in Europe, 2380 kg in the USA and 0.4 kg in Japan. More than 99% of the annual production volume in the USA is accounted for by 4-methyl-*cis*-2-pentene (No. 2194). Half of the annual production volume in Japan is accounted for by 1-nonene (No. 2195). The volume of the annual production in Europe is equally accounted for by 1-octene (No. 2191) and 2,4-nonadiene (No. 2192).

Dietary exposures were estimated using both the SPET and the MSDI method. The highest estimated dietary exposure for each flavouring agent is reported in Table 1. The estimated dietary exposure is highest for 4-methyl-*cis*-2-pentene (No. 2194) (263 µg/day, MSDI value). For the other flavouring agents, the estimated dietary exposures, calculated using either the SPET or

Table 1

Summary of the results of the safety evaluations of aliphatic and alicyclic hydrocarbons used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	Comments on predicted metabolism	Conclusion based on current estimated dietary exposure
Structural class I					
1-Octene	2191	111-66-0 	No, SPET: 3	Note 1	No safety concern
2,4-Nonadiene	2192	56700-78-8 	No, SPET: 13	Note 1	No safety concern
4-Methyl-*cis*-2-pentene	2194	691-38-3 	No, MSDI: 263	Notes 1 and 2	No safety concern
1-Nonene	2195	124-11-8 	No, SPET: 0.6	Note 1	No safety concern
1,3,5,7-Undecatetraene	2196	116963-97-4 	No, SPET: 0.6	Note 1	No safety concern
Mixture of methyl cyclohexadiene and methylene cyclohexene	2197	30640-46-1; 1888-90-0 	No, SPET: 3	Notes 1 and 3	No safety concern

[a] Twenty flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *173*).

[b] *Step 1*: The six flavouring agents in this group are in structural class I.

[c] *Step 2*: The six flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class I is 1800 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case, except for No. 2194, for which the MSDI estimate was higher.

Notes:

1. Epoxidation followed by hydrolysis to yield the corresponding diol, which is conjugated with glucuronic acid and eliminated in the urine.
2. Side-chain oxidation followed by subsequent conjugation with glycine, glucuronic acid or glutathione.
3. Allylic oxidation, epoxidation followed by hydrolysis to yield diols or by ring cleavage followed by conjugation with glucuronic acid and elimination in the urine.

the MSDI method, range from 0.01 to 13 μg/day, with the SPET yielding the highest estimates.

Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of the flavouring agents belonging to the group of aliphatic and alicyclic hydrocarbons has previously been described in the monograph from the sixty-third meeting (Annex 1, reference *174*). Additional information on the metabolism of 1-octene (No. 2191) was available for this meeting.

Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned all six flavouring agents to structural class I *(6)*.

Step 2. All of the flavouring agents in this group are predicted to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the A-side of the Procedure.

Step A3. The highest estimated dietary exposures to all six flavouring agents in this group are below the threshold of concern (i.e. 1800 μg/day for class I). The Committee therefore concluded that these flavouring agents would not pose a safety concern at current estimated dietary exposures.

Table 1 summarizes the evaluations of the six flavouring agents belonging to this group of aliphatic and alicyclic hydrocarbons (Nos 2191, 2192 and 2194–2197).

Consideration of combined intakes from use as flavouring agents

The six additional flavouring agents in this group of aliphatic and alicyclic hydrocarbons have MSDI values of 0.01–263 μg/day. The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

Consideration of secondary components

One flavouring agent in this group (No. 2192) has a minimum assay value of less than 95% (see Annex 3). The secondary components of 2,4-nonadiene (No. 2192) are 1,3-nonadiene, 2,6-nonadiene and 2,7-nonadiene. These compounds, which are structurally related to No. 2192, are considered not to present a safety concern at estimated dietary exposures from use of No. 2192 as a flavouring agent.

Conclusion

In the previous evaluation of flavouring agents in this group of aliphatic and alicyclic hydrocarbons, studies of biochemistry, acute toxicity, short-term and long-term toxicity and genotoxicity were available (Annex 1, reference *174*). None of the 20 flavouring agents of this group raised safety concerns.

For the present evaluation, biochemical data were available for one flavouring agent in this group (No. 2191). For previously evaluated flavouring agents in this group, a study of acute toxicity (No. 1324), studies of short-term toxicity (Nos 1327 and 1324), studies of long-term toxicity (No. 1327) and studies of genotoxicity (Nos 1324, 1327, 1329, 1336, 1339 and 1341) were available. Also, a study of acute toxicity was available for methyl cyclohexadiene, a constituent of No. 2197, which also contains the structurally related methylene cyclohexene. The studies available for the present evaluation support the previous safety evaluations.

The Committee concluded that these six flavouring agents, which are additions to the group of aliphatic and alicyclic hydrocarbons evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

An addendum to the toxicological monograph was prepared.

4.1.2 Aliphatic and aromatic ethers

Introduction

The Committee evaluated three flavouring agents belonging to the group of aliphatic and aromatic ethers. The flavouring agents included two cyclic ethers (Nos 2137 and 2189) and one phenyl ether (No. 2190). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). One of these flavouring agents (No. 2137) was previously evaluated by the Committee at its seventy-sixth meeting (Annex 1, reference *211*). The two others (Nos 2189 and 2190) have not previously been evaluated by the Committee.

The Committee previously evaluated 29 other members of this group of flavouring agents at its sixty-first meeting (Annex 1, reference *166*). The Committee concluded that all 29 flavouring agents in that group were of no safety concern at estimated dietary exposures.

The Committee also evaluated 10 additional members of this group of flavouring agents at its seventy-sixth meeting (Annex 1, reference *212*). The Committee concluded that nine of these flavouring agents were of no safety concern at estimated dietary exposures. For one flavouring agent, nerolidol oxide (No. 2137), additional data were required to complete the evaluation.

None of the flavouring agents in this group has been reported to occur naturally in food.

Assessment of dietary exposure

The total annual volumes of production of the three aliphatic and aromatic ethers are approximately 20 kg in the USA and 0.3 kg in Japan. Approximately 75% of the total annual volume of production in the USA is accounted for by one flavouring agent in this group – 1-cyclopropanemethyl-4-methoxybenzene (No. 2190).

Dietary exposures were estimated using the MSDI method and the SPET. The highest estimated dietary exposure for each flavouring agent is reported in Table 2. The estimated daily dietary exposure is highest for nerolidol oxide (No. 2137) (2500 µg/day, the SPET value obtained from frozen dairy products). For the other flavouring agents, daily dietary exposures ranged from 0.08 to 1250 µg/day, with the SPET yielding the highest estimate in each case.

Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of flavouring agents belonging to the group of aliphatic and aromatic ethers has previously been described (Annex 1, references *167* and *212*). No additional information was available for this meeting.

Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned all three flavouring agents (Nos 2137, 2189 and 2190) to structural class III *(6)*.

Step 2. All three flavouring agents in this group are predicted to be metabolized to innocuous products. The evaluation of these substances therefore proceeded via the A-side of the Procedure.

Step A3. The highest estimated dietary exposures to all three flavouring agents are above the threshold of concern (i.e. 90 µg/day for class III). Accordingly, the evaluation of all three flavouring agents proceeded to step A4.

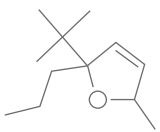
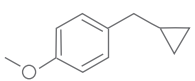
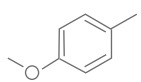
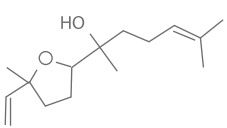
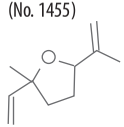
Step A4. None of the three flavouring agents or their metabolites are endogenous substances. Accordingly, the evaluation of all three flavouring agents proceeded to step A5.

Step A5. For cassyrane (No. 2189), a NOAEL of 50 mg/kg bw per day from a 28-day oral gavage study in rats provides an MOE of 2400 in relation to the highest estimated dietary exposure to No. 2189 (SPET = 1250 µg/day or 21 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that cassyrane (No. 2189) would not pose a safety concern at current estimated dietary exposures.

For 1-cyclopropanemethyl-4-methoxybenzene (No. 2190), a NOAEL for the structurally related substance *p*-methylanisole (No. 1243) of 40 mg/kg bw per

Table 2

Summary of the results of the safety evaluations of aliphatic and aromatic ethers used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class III								
Cassyrane	2189	871465-49-5 	Yes, SPET: 1250	No	Yes. The NOAEL of 50 mg/kg bw per day for cassyrane in a 28-day oral gavage study in rats is at least 2400 times the estimated daily dietary exposure to No. 2189 when used as a flavouring agent.	Note 1	–	No safety concern
1-Cyclopropane-methyl-4-methoxybenzene	2190	16510-27-3 	Yes, SPET: 360	No	Yes. The NOAEL of 40 mg/kg bw per day for the structurally related *p*-methylanisole in a 28-day oral gavage study in rats is at least 6700 times the estimated daily dietary exposure to No. 2190 when used as a flavouring agent.	Notes 2 and 3	*p*-Methylanisole (No. 1243) 	No safety concern
Nerolidol oxide	2137	1424-83-5 	Yes, SPET: 2500	No	Yes. The NOAEL of 103 mg/kg bw per day for the structurally related anhydrolinalool oxide in a 90-day dietary study in rats is at least 2500 times the estimated daily dietary exposure to No. 2137 when used as a flavouring agent.	Note 1	Anhydrolinalool oxide (No. 1455) 	No safety concern

[a] The evaluations of 38 of 39 flavouring agents in this group were previously completed by the Committee (Annex 1, references *166* and *211*).

[b] *Step 1*: All three flavouring agents are in structural class III.

[c] *Step 2*: All three flavouring agents are expected to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class III is 90 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The MOE was calculated based on the estimated dietary exposure calculated by the SPET.

Notes:

1. Oxidized by cytochrome P450 isoenzymes to polar metabolites, followed by conjugation with glucuronic acid and elimination in the urine.
2. Metabolized by cytochrome P450–mediated *O*-demethylation.
3. Metabolized by cytochrome P450–mediated ring hydroxylation.

day from a 28-day oral gavage study in rats provides an MOE of 6700 in relation to the highest estimated dietary exposure to No. 2190 (SPET = 360 μg/day or 6 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 1-cyclopropanemethyl-4-methoxybenzene (No. 2190) would not pose a safety concern at current estimated dietary exposures.

For nerolidol oxide (No. 2137), a NOAEL for the structurally related substance anhydrolinalool oxide (No. 1455) of 103 mg/kg bw per day from a 90-day dietary study in rats provides an MOE of 2500 in relation to the highest estimated dietary exposure to No. 2137 (SPET = 2500 μg/day or 42 μg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that nerolidol oxide (No. 2137) would not pose a safety concern at current estimated dietary exposures.

Table 2 summarizes the evaluations of the three flavouring agents belonging to the group of aliphatic and aromatic ethers (Nos 2137, 2189 and 2190).

Consideration of combined intakes from use as flavouring agents

The three flavouring agents in this group of aliphatic and aromatic ethers have low MSDI values (0.08–2 μg/day). The Committee concluded that consideration of combined intakes is not necessary, because these flavouring agents would not contribute significantly to the combined intake of this group.

Conclusion

In the previous evaluations of flavouring agents in the group of aliphatic and aromatic ethers, studies of metabolism and acute toxicity, short-term and long-term studies of toxicity and studies of genotoxicity were available (Annex 1, references *167* and *212*). For one flavouring agent, nerolidol oxide (No. 2137), additional data were required to complete the evaluation.

For the present evaluation, additional acute toxicity studies (Nos 2189 and 2190), short-term studies of toxicity (No. 2189) and genotoxicity studies (Nos 2189 and 2190) were available. For previously evaluated flavouring agents, additional studies on short-term toxicity (Nos 1234 and 1237) and genotoxicity (No. 1237) were available.

The Committee concluded that these three flavouring agents, two of which are additions to the group of aliphatic and aromatic ethers evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

An addendum to the toxicological monograph was prepared.

4.1.3 Ionones and structurally related substances

Introduction

The Committee evaluated three additional flavouring agents belonging to the group of ionones and structurally related substances. The additional flavouring agents included an ionone, β-isomethylionone (No. 2186); an acyclic ionone analogue, pseudoionone (No. 2187); and a damascone, *trans*-α-damascone (No. 2188). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee previously evaluated allyl-α-ionone at its twenty-fourth meeting (Annex 1, reference *53*) and concluded that there were inadequate data to establish an ADI. A further three members of the group were considered at the twenty-eighth meeting (Annex 1, reference *66*), when a group ADI of 0–0.1 mg/kg bw was established. At the Committee's fifty-first meeting (Annex 1, reference *137*), an additional 21 members of this group of flavouring agents were considered. For 20 of these flavouring agents, the Committee concluded that they would not give rise to safety concerns based on the estimated dietary exposures. The remaining substance (No. 402) was not considered to be sufficiently similar to the structural characteristics of the group and was not evaluated further.

One of the three flavouring agents considered at the current meeting, pseudoionone (No. 2187), is a natural component of food and has been detected in liquorice, yerba maté tea, passionfruit juice, tamarind, Chinese microbial-fermented tea and tomato at levels up to 5 mg/kg.

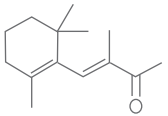
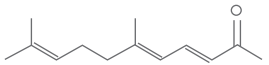
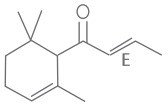
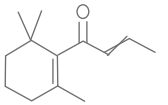
Assessment of dietary exposure

The total annual volumes of production of the three flavouring agents belonging to the group of ionones and structurally related substances are 1 kg in Europe, 3 kg in the USA and 1 kg in Japan. More than 66% of the annual production volume in the USA is accounted for by pseudoionone (No. 2187). More than 99% of the annual production volume in Europe and Japan is accounted for by *trans*-α-damascone (No. 2188).

Dietary exposures were estimated using both the SPET and the MSDI method, with the highest values reported in Table 3. The highest estimated dietary exposure is for pseudoionone (No. 2187) (1000 μg/day, the SPET value obtained from gelatines and puddings). For the other flavouring agents, dietary exposures as SPET or MSDI estimates range from 0.01 to 600 μg/day, with the SPET yielding the highest estimate in each case.

Table 3

Summary of the results of the safety evaluations of ionones and structurally related substances used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3/B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step B4*[e] Adequate margin of exposure for the flavouring agent or related substances?	*Step B5* Do the conditions of use result in an intake >1.5 µg/day?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class I								
β-Isomethylionone	2186	79-89-0 	A3: No, SPET: 600	NR	NR	Notes 1 and 2	–	No safety concern
Pseudoionone	2187	141-10-6 	A3: No, SPET: 1000	NR	NR	Note 2	–	No safety concern
trans-α-Damascone	2188	24720-09-0 	B3: No, SPET: 600	The NOAEL of 2 mg/kg bw per day for the structurally related β-damascone (No. 384) in a 90-day study in rats is 200 times the SPET estimate and 500 000 times the MSDI when No. 2188 is used as a flavouring agent.	Yes	Note 1	β-Damascone (No. 384) 	Additional data required to complete evaluation

NR: not required for evaluation because consumption of the flavouring agent was determined to be of no safety concern at step A3 of the Procedure

[a] Twenty flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *137*).

[b] *Step 1*: The three flavouring agents are in structural class I (Nos 2186–2188).

[c] *Step 2*: Nos 2186 and 2187 can be predicted to be metabolized to innocuous products. No. 2188 cannot be predicted to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class I is 1800 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The MOEs were calculated based on the estimated dietary exposure calculated using the SPET. In cases where the resulting MOE was relatively low, a comparison with the MSDI was also made.

Notes:

1. Metabolized primarily via ring oxidation to 3-oxo and 3-hydroxy derivatives, which are eliminated.
2. Metabolized primarily through reduction of the ketone followed by conjugation with glucuronic acid; alternatively, Michael addition to the alkene group by glutathione followed by elimination as the mercapturic acid conjugate.

Absorption, distribution, metabolism and elimination

The absorption, metabolism and elimination of flavouring agents of the ionones and structurally related substances group have previously been described (Annex 1, reference *138*). Orally administered ionones are absorbed and metabolized in mammals by allylic hydroxylation of the ring followed by oxidation of the hydroxyl group to corresponding ketone derivatives. Reduction of the ketone function to the corresponding secondary alcohol also occurs. Combinations of these detoxication reactions result in the formation of multiple polar metabolites that are excreted in the urine unchanged or conjugated with glucuronic acid.

Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned all three flavouring agents (Nos 2186, 2187 and 2188) to structural class I *(6)*.

Step 2. Two of the flavouring agents (Nos 2186 and 2187) in this group are predicted to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the A-side of the Procedure. The other flavouring agent (No. 2188) in this group cannot be predicted to be metabolized to innocuous products. Therefore, the evaluation of this flavouring agent proceeded via the B-side of the Procedure.

Step A3. The highest estimated daily dietary exposures for each of the two flavouring agents in structural class I that are predicted to be metabolized to innocuous products (Nos 2186 and 2187) are below the threshold of concern (i.e. 1800 µg/day for class I). The Committee therefore concluded that neither of the two flavouring agents would pose a safety concern at current estimated dietary exposures.

Step B3. The highest estimated daily dietary exposure for the flavouring agent in structural class I that is not predicted to be metabolized to innocuous products (No. 2188) is below the threshold of concern (i.e. 1800 µg/day for class I). Accordingly, the evaluation of this flavouring agent proceeded to step B4.

Step B4. For *trans*-α-damascone (No. 2188), the NOAEL of 2 mg/kg bw per day for the structurally related β-damascone (No. 384) in a 90-day study in rats is 200 times the SPET estimate (600 µg/day or 10 µg/kg bw per day) and 500 000 times the MSDI (0.3 µg/day or 0.004 µg/kg bw per day) when No. 2188 is used as a flavouring agent. The Committee therefore concluded that the NOAEL does not provide an adequate MOE based on the SPET, and the evaluation proceeded to step B5.

Step B5. The conditions of use result in an intake greater than 1.5 µg/day. Therefore, the Committee concluded that additional data are required to complete the evaluation.

Table 3 summarizes the evaluations of the three ionones and structurally related substances used as flavouring agents (Nos 2186, 2187 and 2188) in this group.

Consideration of combined intakes from use as flavouring agents

The two additional flavouring agents in this group of ionones and structurally related substances that were concluded to be of no safety concern have low MSDI values (0.01–0.2 μg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this group.

Conclusion

In the previous evaluation of flavouring agents in this group of ionones and structurally related substances, studies of acute toxicity, short-term toxicity and genotoxicity were available (Annex 1, reference *138*). None of the 20 previously evaluated flavouring agents raised safety concerns.

For the present evaluation, studies of acute toxicity (No. 2187), studies of genotoxicity (Nos 2187 and 2188) and a study of developmental toxicity (No. 2187) were available. For previously evaluated flavouring agents, there were additional studies of acute toxicity (Nos 388, 389, 394, 399 and 404), studies of short-term toxicity (No. 404), studies of genotoxicity (Nos 386–389, 394, 401, 403 and 404) and studies of developmental toxicity (Nos 389 and 404). The additional data provided supported the previous safety evaluations.

The Committee concluded that two of these three flavouring agents (Nos 2186 and 2187), which are additions to the group of ionones and structurally related substances evaluated previously, would not give rise to safety concerns at current estimated dietary exposures. For *trans*-α-damascone (No. 2188), the Committee requires additional toxicological and/or dietary exposure information in order to complete the evaluation. The Committee was aware of additional genotoxicity data reporting equivocal results for a structurally related damascone; therefore, information to address any concerns regarding potential genotoxicity should also be provided.

An addendum to the toxicological monograph was prepared.

4.1.4 Miscellaneous nitrogen-containing substances

Introduction

The Committee evaluated two additional flavouring agents belonging to the group of miscellaneous nitrogen-containing substances. These flavouring agents were a uridic diamide with additional ester functionality and an alicyclic alkyl side-chain (No. 2203) and an aminoquinoline carboxylic acid derivative containing an aliphatic amide side-chain (No. 2204). The evaluations were conducted using

the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). The two flavouring agents have not previously been evaluated by the Committee, and both are reported to be flavour modifiers.

The Committee previously evaluated 16 other members of this group of flavouring agents at the sixty-fifth meeting (Annex 1, reference *178*), 14 other members of this group at the sixty-ninth meeting (Annex 1, reference *190*) and 2 other members of this group at the seventy-sixth meeting (Annex 1, reference *211*). The Committee concluded that all 32 of these flavouring agents were of no safety concern at estimated dietary exposures.

Neither of the additional flavouring agents in this group (Nos 2203 and 2204) has been reported to occur naturally in food.

Assessment of dietary exposure

The total annual volume of production for the two flavouring agents belonging to the group of miscellaneous nitrogen-containing substances is 10 kg in the USA, with no reported data from Europe or Japan. Ninety per cent of the annual production volume in the USA is accounted for by 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204).

Dietary exposures were estimated using both the SPET and the MSDI method, with highest values reported in Table 4. The estimated dietary exposure is highest for 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) (2400 μg/day, the SPET value obtained from non-alcoholic beverages). For the other flavouring agent, 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203), the MSDI was 0.1 μg/day, and the SPET value was 800 μg/day.

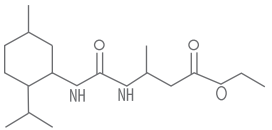
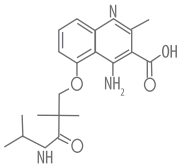
Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of the flavouring agents belonging to the group of miscellaneous nitrogen-containing substances has previously been described in the monographs of the sixty-fifth, sixty-ninth and seventy-sixth meetings (Annex 1, references *179*, *191* and *212*).

Two common metabolic pathways are expected to be involved in the metabolism of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203). As an ethyl ester, it will be hydrolysed by carboxylesterases in gastric juice, intestinal fluid and hepatocytes. Released carboxylic acids will be excreted in urine either free or conjugated with glucuronic acid or glycine. As an alkyl *N*-substituted urea, No. 2203 is also expected to be oxidized at the α-carbon group by cytochrome P450 enzymes to generate an unstable carbinol urea, which is expected to release the corresponding ketones, menthone and 3-oxobutyric acid ethyl ester.

Table 4

Summary of the results of the safety evaluations of miscellaneous nitrogen-containing substances used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Follow-on from step B3*[e] Are additional data available for the flavouring agent with an estimated dietary exposure exceeding the threshold of concern?	Comments on predicted metabolism	Conclusion based on current estimated dietary exposure
Structural class III						
3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester	2203	1160112-20-8 	Yes, SPET: 800	Yes. The NOAEL of 776.5 mg/kg bw per day in a 90-day study in rats is 60 000 times the estimated dietary exposure to No. 2203 when used as a flavouring agent.	Note 1	No safety concern
4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropox)-2-methylquinoline-3-carboxylic acid (and its hemisulfate monohydrate salt)	2204	1359963-68-0 	Yes, SPET: 2400	Yes. The NOAEL of 100 mg/kg bw per day in a 90-day study in rats is 2500 times the estimated dietary exposure to No. 2203 when used as a flavouring agent.	Note 2	No safety concern

[a] Thirty-two flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *178*, *190* and *211*).

[b] *Step 1*: The two flavouring agents in this group (Nos 2203 and 2204) are in structural class III.

[c] *Step 2*: Neither of the flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class III is 90 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest daily dietary exposures calculated by either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The MOEs were calculated based on the estimated dietary exposure calculated by the SPET.

Notes:

1. The ester functionality will readily hydrolyse to the corresponding acid and ethanol, which will form conjugates with glucuronic acid and be readily eliminated. The ureido functionality will be oxidized by cytochrome P450 and form the α-carbinol. The carbinol urea is expected to release urea and the corresponding ketones, menthone and 3-oxobutyric acid ethyl ester.
2. Primarily excreted in the faeces unchanged, with very minor amounts of oxidative metabolites eliminated in the urine.

Metabolic studies for 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) have shown that this compound and its hemisulfate monohydrate salt have low oral bioavailability in rats. Once absorbed, the parent compound is poorly metabolized, being primarily excreted unchanged in the faeces, with only 0.1–0.6% eliminated in the urine as possible metabolites. In a study in which human or rat liver microsomes were incubated with 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204) for 60 minutes, less than 0.1% was converted to oxidative metabolites. Thus, either very little or no Phase 1 metabolism occurred.

Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned both flavouring agents (Nos 2203 and 2204) to structural class III *(6)*.

Step 2. Neither of the flavouring agents in this group (Nos 2203 and 2204) can be predicted to be metabolized to innocuous products. The evaluation of both of these flavouring agents proceeded via the B-side of the Procedure.

Step B3. The highest estimated dietary exposures for both flavouring agents in structural class III are above the threshold of concern (i.e. 90 μg/day for class III). Accordingly, data must be available on the flavouring agent or a closely related substance in order to perform a safety evaluation.

Consideration of flavouring agents with high exposure evaluated via the B-side of the decision-tree:

In accordance with the Procedure, additional data were evaluated for 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203) and 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204), as their estimated dietary exposures exceeded the threshold of concern for structural class III (90 μg/day).

For 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203), a genotoxicity study and a 90-day toxicity study were available. This flavouring agent was negative for bacterial mutagenesis with and without exogenous activation. The NOAEL of 776.5 mg/kg bw per day (the highest dose tested) in a 90-day study in rats provides an MOE of 60 000 (SPET = 800 μg/day or 13 μg/kg bw per day) when No. 2203 is used as a flavouring agent. The Committee concluded that, on the basis of all of the available evidence, 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203) would not pose a safety concern at current estimated dietary exposures.

For 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (No. 2204), pharmacokinetic data and 28-day and 90-day toxicity, genotoxicity and developmental toxicity studies were available. This flavouring agent was found to be poorly bioavailable and rapidly excreted in the faeces unchanged. A NOAEL of 100 mg/kg bw per day was identified in a 28-day and a 90-day study in rats. The flavouring agent was negative for bacterial mutagenesis with and without an exogenous activation system, for clastogenicity in human blood lymphocytes with and without an exogenous activation system and for induction of micronuclei in mouse bone marrow erythrocytes. The developmental toxicity study in rats had a NOAEL of 1000 mg/kg bw per day, the highest dose tested. The NOAEL of 100 mg/kg bw per day in the 90-day study in rats provides an MOE of 2500 (SPET = 2400 µg/day or 40 µg/kg bw per day) when No. 2204 is used as a flavouring agent. The Committee concluded that, on the basis of all of the available evidence, 4-amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid and its hemisulfate monohydrate salt would not pose a safety concern at current estimated dietary exposures.

Table 4 summarizes the evaluations of the two flavouring agents belonging to the group of miscellaneous nitrogen-containing substances (Nos 2203 and 2204).

Consideration of combined intakes from use as flavouring agents

The two additional flavouring agents in this group of miscellaneous nitrogen-containing substances have low MSDI values (0.1–1 µg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

Conclusion

In the previous evaluations of flavouring agents in this group of miscellaneous nitrogen-containing substances, studies of acute toxicity, short-term toxicity and genotoxicity were available (Annex 1, references *179*, *191* and *212*).

For the present evaluation, additional biochemical data, short-term studies of toxicity (28–90 days), in vitro and in vivo genotoxicity studies and a developmental toxicity study were available for the two additional flavouring agents belonging to this group (Nos 2203 and 2204).

The Committee concluded that these two flavouring agents, which are additions to the group of miscellaneous nitrogen-containing substances evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

An addendum to the toxicological monograph was prepared.

4.1.5 Monocyclic and bicyclic secondary alcohols, ketones and related esters

Introduction

The Committee evaluated four additional flavouring agents belonging to the group of monocyclic and bicyclic secondary alcohols, ketones and related esters. The additional flavouring agents included one bicyclic secondary alcohol, 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-ol (No. 2198); and three bicyclic ketones, *dl*-camphor (No. 2199), *l*-fenchone (No. 2200) and 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-one (No. 2201). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). None of these flavouring agents has previously been evaluated by the Committee.

The Committee previously evaluated 32 other members of this group of flavouring agents at its sixty-third meeting (Annex 1, reference *173*). The Committee concluded that all 32 flavouring agents in that group were of no safety concern at estimated dietary exposures.

The Committee also evaluated another nine members of this group of flavouring agents at its sixty-ninth meeting (Annex 1, reference *190*). The Committee concluded that all nine additional flavouring agents were of no safety concern at estimated dietary exposures.

None of the flavouring agents in this group has been reported to occur as a natural component of food.

Assessment of dietary exposure

The total annual volumes of production of the four flavouring agents belonging to the group of monocyclic and bicyclic secondary alcohols, ketones and related esters are 491 kg in Europe, 0.3 kg in the USA and 30 kg in Japan. More than 99% of the total annual volumes of production in Europe and Japan is accounted for by *dl*-camphor (No. 2199).

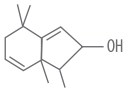
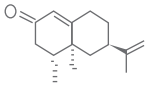
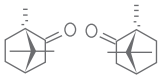
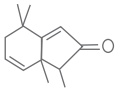
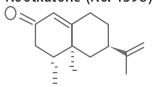
Dietary exposures were estimated using both the SPET and the MSDI method, with the highest values reported in Table 5. The estimated dietary exposures are highest for 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-ol (No. 2198) and 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-one (No. 2201) (625 µg/day, the SPET value obtained from jams and jellies). For the other flavouring agents, the estimated dietary exposures range from 0.01 to 150 µg/day, with the SPET yielding the highest estimates in all cases.

Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of flavouring agents of the monocyclic and bicyclic secondary alcohols, ketones and related esters group has previously been described in the reports of the sixty-

Table 5

Summary of the results of the safety evaluations of monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class II								
2,2,6,7-Tetramethyl-bicyclo[4.3.0]nona-4,9(1)-dien-8-ol	2198	97866-86-9 	Yes, SPET: 625	No	Yes. The NOAEL of 10 mg/kg bw per day for the structurally related nootkatone in a 28-day study in rats is 1000 times the estimated dietary exposure to No. 2198 when used as a flavouring agent.	Notes 1 and 2	Nootkatone (No. 1398) 	No safety concern
dl-Camphor	2199	76-22-2 	No, SPET: 150	No	NR	Notes 1, 2 and 3	–	No safety concern
l-Fenchone	2200	7787-20-4 	No, SPET: 3	No	NR	Notes 1, 2 and 3	–	No safety concern
2,2,6,7-Tetramethyl-bicyclo[4.3.0]nona-4,9(1)-dien-8-one	2201	97844-16-1 	Yes, SPET: 625	No	Yes. The NOAEL of 10 mg/kg bw per day for the structurally related nootkatone in a 28-day study in rats is 1000 times the estimated dietary exposure to No. 2201 when used as a flavouring agent.	Notes 1, 2 and 3	Nootkatone (No. 1398) 	No safety concern

NR: Not required for evaluation because consumption of the flavouring agent was determined to be of no safety concern at step A3 of the Procedure

[a] Forty-one flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *173* and *190*).

[b] *Step 1:* The four flavouring agents in this group (Nos 2198–2201) are in structural class II.

[c] *Step 2:* All four flavouring agents in this group can be predicted to be metabolized to innocuous products.

[d] The threshold for human dietary exposure for structural class II is 540 µg/day. All dietary exposure values are expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated by either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The MOEs were calculated based on the estimated dietary exposure calculated by the SPET.

Notes:

1. Formation of glucuronic acid conjugates directly or after metabolism, which are subsequently eliminated in the urine.
2. Cytochrome P450–mediated oxidation of alkyl ring substituents and ring positions.
3. Reduced to yield the corresponding alcohol.

third and sixty-ninth meetings (Annex 1, references *174* and *191*). New data on a representative member of this group show 20% bioavailability of orally administered D-borneol (No. 1385) in mice.

Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned all four flavouring agents (Nos 2198–2201) to structural class II *(6)*.

Step 2. All four flavouring agents in this group are predicted to be metabolized to innocuous products. Therefore, the evaluation of all of these flavouring agents proceeded via the A-side of the Procedure.

Step A3. The estimated dietary exposures for two of the flavouring agents (Nos 2199 and 2200) in structural class II are below the threshold of concern (i.e. 540 µg/day for class II). According to the Procedure, the safety of these flavouring agents raises no concern at current estimated dietary exposures. The estimated dietary exposures for two flavouring agents (Nos 2198 and 2201) are above the threshold of concern for structural class II (i.e. 540 µg/day for class II). Accordingly, the evaluation of these flavouring agents proceeded to step A4.

Step A4. These flavouring agents (Nos 2198 and 2201) and their metabolites are not endogenous, and therefore their evaluations proceeded to step A5.

Step A5. For 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-ol (No. 2198), the NOAEL of 10 mg/kg bw per day for the structurally related substance nootkatone (No. 1398) obtained in a 28-day study in rats provides an adequate MOE of 1000 in relation to the estimated dietary exposure to No. 2198 (SPET = 625 µg/day or 10 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-ol (No. 2198) would not pose a safety concern at current estimated dietary exposures.

For 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-one (No. 2201), the NOAEL of 10 mg/kg bw per day for the structurally related substance nootkatone (No. 1398) obtained in a 28-day study in rats provides an adequate MOE of 1000 in relation to the estimated dietary exposure to No. 2201 (SPET = 625 µg/day or 10 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that 2,2,6,7-tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-one (No. 2201) would not pose a safety concern at current estimated dietary exposures.

Table 5 summarizes the evaluations of the four monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents (Nos 2198–2201) in this group.

Consideration of combined intakes from use as flavouring agents

The four additional flavouring agents in this group of monocyclic and bicyclic secondary alcohols, ketones and related esters have low MSDI values (0.01–41 µg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this group.

Conclusion

In the previous evaluations of flavouring agents in this group of monocyclic and bicyclic secondary alcohols, ketones and related esters, studies of acute toxicity, short-term and long-term toxicity and genotoxicity were available. For previously evaluated substances of this group, additional biochemical data were available at this meeting (No. 1385); a short-term study of toxicity (No. 1867), studies of genotoxicity (Nos 1385 and 1867) and a study of reproductive and developmental toxicity (No. 1388) were also available. The toxicity data available for this evaluation supported those from the previous evaluations (Annex 1, references *174* and *191*).

The Committee concluded that these four flavouring agents (Nos 2198–2201), which are additions to the group of monocyclic and bicyclic secondary alcohols, ketones and related esters, would not give rise to safety concerns at current estimated dietary exposures.

An addendum to the toxicological monograph was prepared.

4.1.6 Phenol and phenol derivatives

Introduction

The Committee evaluated four flavouring agents belonging to the group of phenol and phenol derivatives. The additional flavouring agents included a flavanone (No. 2207), a dihydrochalcone (No. 2208), a polyphenol (No. 2209) and a lignan (No. 2210). The evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). These four flavouring agents have not previously been evaluated by the Committee, and all are reported to be flavour modifiers.

The Committee previously evaluated 48 other members of this group of flavouring agents at its fifty-fifth meeting (Annex 1, reference *150*), 13 other members at its seventy-third meeting (Annex 1, reference *203*) and 3 additional members at its seventy-sixth meeting (Annex 1, reference *212*). The Committee concluded that all 64 flavouring agents were of no safety concern at estimated dietary exposures.

Two of the four flavouring agents (Nos 2207 and 2210) in this group have been reported to occur naturally and can be found in a broad variety of

fruits, vegetables, grains, nuts, seeds, coffee and tea, in addition to many other foods.

Assessment of dietary exposure

The total annual volumes of production of the four flavouring agents in the phenol and phenol derivatives group are approximately 1 kg in the USA and 721 kg in Japan. Approximately 96% of the total annual volume of production in Japan is accounted for by one flavouring agent in this group, myricitrin (No. 2207).

Dietary exposures were estimated using the SPET and the MSDI method, with the highest values reported in Table 6. The estimated dietary exposure is highest for (–)-matairesinol (No. 2210) (7500 μg/day, the SPET value for non-alcoholic beverages). For the other flavouring agents, the dietary exposures ranged from 0.03 to 6000 μg/day, with the SPET yielding the highest estimate in each case.

Absorption, distribution, metabolism and elimination

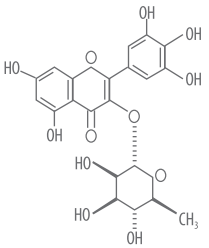
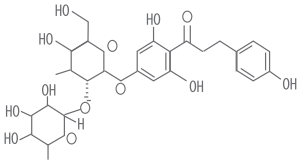
Information on the absorption, distribution, metabolism and elimination of the flavouring agents belonging to the phenol and phenol derivatives group has previously been described in the monographs of the fifty-fifth, seventy-third and seventy-sixth meetings (Annex 1, references *150*, *203* and *212*); additional information on the absorption, distribution, metabolism and elimination of polyphenols was also available for this meeting.

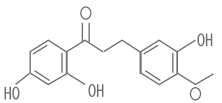
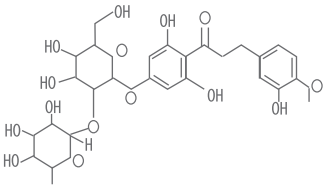
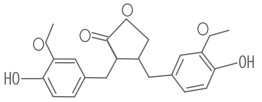
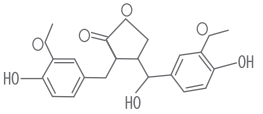
Glycoside conjugates of polyphenols are hydrolysed on the brush border of small intestine epithelial cells or within the epithelial cells. Polyphenols are rapidly but incompletely absorbed after oral administration. Metabolism occurs both in the gastrointestinal tract and after absorption. Absorbed polyphenols are metabolized through hydrolysis, sulfation, glucuronidation and/or methylation. Urinary excretion of parent substance or metabolites is rapid to relatively slow. Biliary excretion also occurs. Metabolites not absorbed in the small intestine may undergo further metabolism in the large intestine. Both glycosylated and aglycone metabolites may be excreted in the faeces. The microflora may also cleave conjugated moieties, with the resultant aglycones undergoing ring fission, leading to phenolic acid and cinnamic acid derivatives. These metabolites may be absorbed and ultimately excreted in the urine.

Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned one flavouring agent (No. 2207) to structural class II and three flavouring agents (Nos 2208–2210) to structural class III *(6)*.

Table 6
Summary of the results of the safety evaluations of phenol and phenol derivatives used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class II								
Myricitrin	2207	17912-87-7 	Yes, SPET: 3000	No	Yes. The NOAEL of 884 mg/kg bw per day in a 52-week study in rats is 18 000 times the estimated dietary exposure to No. 2207 when used as a flavouring agent.	Notes 1 and 2	–	No safety concern
Structural class III								
Naringin dihydrochalcone	2208	18916-17-1 	Yes, SPET: 4000	No	Yes. The NOAEL of 500 mg/kg bw per day in short-term studies in rats is 7500 times the estimated dietary exposure to No. 2208 when used as a flavouring agent.	Notes 1 and 2	–	No safety concern

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step A4* Is the flavouring agent or are its metabolites endogenous?	*Step A5*[e] Adequate margin of exposure for the flavouring agent or related substances?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
1-(2,4-Dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)-propan-1-one	2209	50297-39-7 	Yes, SPET: 6000	No	Yes. The NOAEL of 760 mg/kg bw per day for the structurally related neohesperidin dihydrochalcone in a 90-day study in rats is 7600 times the estimated dietary exposure to No. 2209 when used as a flavouring agent.	Note 2	Neohesperidin dihydrochalcone 	No safety concern
(−)-Matairesinol	2210	580-72-3 	Yes, SPET: 7500	No	Yes. The NOAEL of 160 mg/kg bw per day for the structurally related 7-hydroxy-matairesinol in a 90-day study in rats is 1300 times the estimated dietary exposure to No. 2210 when used as a flavouring agent.	Note 2	7-Hydroxymatairesinol 	No safety concern

[a] Sixty-four flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *149*, *202* and *211*).
[b] *Step 1*: One flavouring agent in this group (No. 2207) is in structural class II. Three flavouring agents in this group (Nos 2208–2210) are in structural class III.
[c] *Step 2*: All four flavouring agents in this group can be predicted to be metabolized to innocuous products.
[d] The thresholds for human dietary exposure for structural classes II and III are 540 µg/day and 90 µg/day, respectively. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest values calculated by either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.
[e] The MOEs were calculated based on the estimated dietary exposure calculated using the SPET.

Notes:

1. Phenolic glucosides are expected to undergo hydrolysis via epithelial cells or gut microflora to the corresponding phenol (aglycone).
2. Phenols are methylated or form sulfates or glucuronides prior to elimination in the urine or faeces.

Step 2. All four flavouring agents in this group can be predicted to be metabolized to innocuous products. The evaluation of all of these flavouring agents therefore proceeded via the A-side of the Procedure.

Step A3. The highest estimated dietary exposures for all four flavouring agents are above the thresholds of concern (i.e. 540 µg/day for class II, 90 µg/day for class III). Accordingly, the evaluation of all four flavouring agents proceeded to step A4.

Step A4. None of the four flavouring agents or their metabolites are endogenous substances. Accordingly, the evaluation of all four flavouring agents proceeded to step A5.

Step A5. For myricitrin (No. 2207), the NOAEL of 884 mg/kg bw per day from a 52-week study in rats provides an adequate MOE of 18 000 in relation to the highest estimated dietary exposure (SPET = 3000 µg/day or 50 µg/kg bw per day) to No. 2207 when used as a flavouring agent. The Committee therefore concluded that myricitrin would not pose a safety concern at current estimated dietary exposures.

For naringin dihydrochalcone (No. 2208), the NOAEL of 500 mg/kg bw per day from short-term studies in rats provides an adequate MOE of 7500 in relation to the highest estimated dietary exposure (SPET = 4000 µg/day or 67 µg/kg bw per day) to No. 2208 when used as a flavouring agent. The Committee therefore concluded that naringin dihydrochalcone would not pose a safety concern at current estimated dietary exposures.

For 1-(2,4-dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (No. 2209), the NOAEL of 760 mg/kg bw per day for the structurally related substance neohesperidin dihydrochalcone from a 90-day study in rats provides an adequate MOE of 7600 in relation to the highest estimated dietary exposure (SPET = 6000 µg/day or 100 µg/kg bw per day) to No. 2209 when used as a flavouring agent. The Committee therefore concluded that 1-(2,4-dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one would not pose a safety concern at current estimated dietary exposures.

For (–)-matairesinol (No. 2210), the NOAEL of 160 mg/kg bw per day for the structurally related 7-hydroxymatairesinol in a 90-day study in rats provides an adequate MOE of 1300 in relation to the highest estimated dietary exposure (SPET = 7500 µg/day or 125 µg/kg bw per day) to No. 2210 when used as a flavouring agent. The Committee therefore concluded that (–)-matairesinol would not pose a safety concern at current estimated dietary exposures.

Table 6 summarizes the evaluations of the four flavouring agents belonging to the phenol and phenol derivatives group (Nos 2207–2210).

Consideration of combined intakes from use as flavouring agents

The four flavouring agents in this phenol and phenol derivatives group have MSDI values of 0.03–182 µg/day. The Committee concluded that consideration of combined intakes is not necessary, because these flavouring agents would not contribute significantly to the combined intake of this group.

Conclusion

In the previous evaluations of flavouring agents in the phenol and phenol derivatives group, studies of acute toxicity, short-term and long-term toxicity (18 days to 2 years), carcinogenicity, genotoxicity and reproductive and developmental toxicity were available (Annex 1, references *150*, *203* and *212*).

For the present evaluation, biochemical data, acute, short-term and long-term studies of toxicity and genotoxicity studies were available for one flavouring agent in this group (No. 2207); biochemical data and short-term studies of toxicity were available for one flavouring agent in this group (No. 2208); biochemical data were available for one flavouring agent in this group (No. 2210); and genotoxicity data were available for one flavouring agent in this group (No. 2209). Genotoxicity and developmental toxicity studies were available for 7-hydroxymatairesinol, a structurally related substance. The studies available for the present evaluation support the previous safety evaluations.

The Committee concluded that these four flavouring agents, which are additions to the group of phenol and phenol derivatives evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

An addendum to the toxicological monograph was prepared.

4.1.7 Phenyl-substituted aliphatic alcohols and related aldehydes and esters

Introduction

The Committee evaluated two additional flavouring agents belonging to the group of phenyl-substituted aliphatic alcohols and related aldehydes and esters. The additional flavouring agents included one ester (No. 2202) and one aldehyde (No. 2069), both containing phenyl substituents. Neither of these agents has previously been evaluated by the Committee.

The Committee previously evaluated 22 other members of this group of flavouring agents at its sixty-third meeting (Annex 1, reference *173*). The Committee concluded that all 22 flavouring agents in that group were of no safety concern at estimated dietary exposures.

Ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) has been reported to occur in tonka beans.

Assessment of dietary exposure

The total annual volume of production of ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) is 0.1 kg in the USA, with no reported production volume for Europe or Japan. Dietary exposures were estimated using both the SPET and the MSDI method. The highest estimated dietary exposure for ethyl 3-(2-hydroxyphenyl)-propanoate (No. 2202) is 100 μg/day, the SPET value obtained from milk products (see Table 7).

The total annual volume of production of (±)-2-phenyl-4-methyl-2-hexenal (No. 2069) is 0.1 kg in Japan, with no reported production volume for the USA or Europe. The highest estimated dietary exposure for (±)-2-phenyl-4-methyl-2-hexenal (No. 2069) is 150 μg/day, the SPET value obtained from instant coffee and tea.

Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of flavouring agents belonging to the phenyl-substituted aliphatic alcohols and related aldehydes and esters group has previously been described in the monograph of the sixty-third meeting (Annex 1, reference *174*). Additional data on the in vitro metabolism of 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) and 2-phenylpropionaldehyde (No. 1467), flavouring agents structurally similar to No. 2202, were submitted that are in line with the data previously submitted.

Genotoxicity

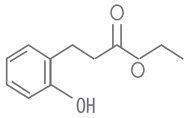
For the current evaluation, additional genotoxicity studies were available for 2-phenyl-2-butenal (No. 1474), a flavouring agent previously evaluated in this group.

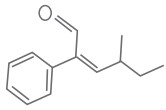
2-Phenyl-2-butenal (No. 1474) did not show mutagenic potential in bacterial reverse mutation assays in the absence or presence of metabolic activation. In an in vitro micronucleus test in cultured human lymphocytes, it showed genotoxic potential only in the absence of metabolic activation. Data from two in vivo micronucleus tests gave inconclusive results. The results of these in vivo studies would have been more convincing had direct evidence of systemic exposure to 2-phenyl-2-butenal been demonstrated. Also, as this substance was genotoxic only without metabolic activation in the in vitro micronucleus test, the Committee concluded that additional data are needed to address these concerns and conclude on the genotoxicity of 2-phenyl-2-butenal (No. 1474) and the other previously evaluated α,β-unsaturated 2-phenyl compounds in this group (Nos 1472, 1473 and 1476) and No. 2069.

The Committee concluded that the Procedure cannot be applied to No. 2069 until the concerns regarding genotoxicity are resolved.

Table 7

Summary of the results of the safety evaluations of phenyl-substituted aliphatic alcohols and related aldehydes and esters used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step A3*[d] Does estimated dietary exposure exceed the threshold of concern?	Comments on predicted metabolism	Conclusion based on current estimated dietary exposure
Structural class I					
Ethyl 3-(2-hydroxyphenyl)-propanoate	2202	20921-04-4 	No, SPET: 100	Notes 1 and 2	No safety concern

Flavouring agent	No.	CAS no. and structure	Conclusion
Flavouring agent not evaluated by the Procedure			
(±)-2-Phenyl-4-methyl-2-hexenal	2069	26643-92-5 	The Procedure cannot be applied to this substance until concerns regarding genotoxicity are addressed

[a] Twenty-two flavouring agents in this group were previously evaluated by the Committee (Annex 1, reference *173*).
[b] *Step 1*: Flavouring agent No. 2202 is in structural class I.
[c] *Step 2*: Flavouring agent No. 2202 can be expected to be metabolized to innocuous products.
[d] The threshold for human dietary exposure for structural class I is 1800 µg/day. The dietary exposure value is expressed in µg/day. The dietary exposure value listed represents the highest estimated dietary exposure calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure.

Notes:

1. Readily forms glucuronic acid conjugates, which are subsequently excreted in the urine.
2. Esters undergo rapid hydrolysis to liberate the corresponding alcohol and carboxylic acid.

Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the additional flavouring agent in this group of phenyl-substituted aliphatic alcohols and related aldehydes and esters, the Committee assigned ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) to structural class I *(6)*.

Step 2. Ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) can be predicted to be metabolized to innocuous products. The evaluation of this flavouring agent therefore proceeded via the A-side of the Procedure.

Step A3. The highest estimated dietary exposure to ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) is below the threshold of concern

(i.e. 1800 µg/day for class I). The Committee therefore concluded that ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202) would not pose a safety concern at current estimated dietary exposures.

Table 7 summarizes the evaluation of the additional flavouring agent belonging to the group of phenyl-substituted aliphatic alcohols and related aldehydes and esters (No. 2202).

Consideration of combined intakes from use as flavouring agents

The additional flavouring agent in this group of phenyl-substituted aliphatic alcohols and related aldehydes and esters that was evaluated according to the Procedure has a low MSDI value (0.01 µg/day). The Committee concluded that consideration of combined intakes is not necessary, because this additional flavouring agent would not contribute significantly to the combined intake of this flavouring group.

Conclusion

In the previous evaluation of flavouring agents in this group of phenyl-substituted aliphatic alcohols and related aldehydes and esters, biochemical studies and studies of acute toxicity, short-term toxicity and genotoxicity were available. The results of those studies did not raise safety concerns.

For the current evaluation, additional studies were available on flavouring agents previously evaluated in this group, including metabolism studies (Nos 1465 and 1467), short-term studies of toxicity (Nos 1465–1467), genotoxicity studies (No. 1474) and a study of reproductive toxicity (No. 1465).

The metabolism and toxicity data available for this evaluation generally supported those from the previous evaluation. However, the new genotoxicity studies on 2-phenyl-2-butenal (No. 1474) raise concerns regarding No. 1474 and the other previously evaluated α,β-unsaturated 2-phenyl compounds in this group (Nos 1472, 1473 and 1476) and No. 2069.

The Committee concluded that the Procedure cannot be applied to No. 2069 until the concerns regarding genotoxicity are resolved. The Committee recommended that the evaluations of the other α,β-unsaturated aldehydes in this group (Nos 1472–1474 and 1476) should be reconsidered at a future meeting, given the potential genotoxicity of 2-phenyl-2-butenal (No. 1474).

The Committee concluded that ethyl 3-(2-hydroxyphenyl)propanoate (No. 2202), which is an addition to the group of phenyl-substituted aliphatic alcohols and related aldehydes and esters evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

An addendum to the toxicological monograph was prepared.

4.1.8 Sulfur-containing heterocyclic compounds

Introduction

The Committee evaluated three flavouring agents belonging to the group of sulfur-containing heterocyclic compounds. The flavouring agents included one dithiazine (No. 2205), one thiazoline (No. 2206) and one thiophene (No. 1051). Two of the flavouring agents (Nos 2205 and 2206) have not been previously evaluated by the Committee, and their evaluations were conducted using the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*). The third flavouring agent (No. 1051) was previously evaluated by the Committee at its fifty-ninth meeting (Annex 1, reference *161*) and was reconsidered by the current Committee because of concerns about potential mutagenicity. The Committee was informed that the flavouring industry is taking steps to remove this compound from the market.

The Committee previously evaluated 30 members of this group of flavouring agents at its fifty-ninth meeting (Annex 1, reference *161*). Based on the data available, the Committee concluded that all 30 members of this group of flavouring agents were of no safety concern at estimated dietary exposures. No. 1051 was a member of this group of flavouring agents.

The Committee also evaluated 17 additional members of this group of flavouring agents at its sixty-eighth meeting (Annex 1, reference *188*) and 12 additional members of this group of flavouring agents at its seventy-sixth meeting (Annex 1, reference *212*). The Committee concluded that all 29 additional flavouring agents were of no safety concern at estimated dietary exposures.

All three flavouring agents have been reported to occur naturally and can be found in shrimp, beef and sesame seed oil.

Assessment of dietary exposure

The annual volume of production of triethylthialdine (No. 2205) is 0.1 kg in Europe, and the annual volume of production of 2-isopropyl-4-methyl-3-thiazoline (No. 2206) is 0.1 kg in the USA.

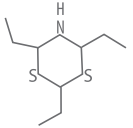
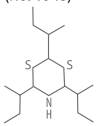
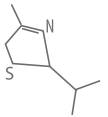
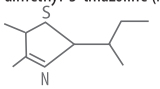
Dietary exposures were estimated for these two flavouring agents using the SPET and the MSDI method, with the highest values reported in Table 8. The estimated dietary exposures for Nos 2205 and 2206 range from 0.01 to 75 µg/day, with the SPET yielding the highest estimates in both cases.

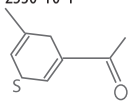
Absorption, distribution, metabolism and elimination

Information on the absorption, distribution, metabolism and elimination of the flavouring agents belonging to the group of sulfur-containing heterocyclic compounds has previously been described in the monographs of the fifty-ninth, sixty-eighth and seventy-sixth meetings (Annex 1, references *161*, *188*

Table 8

Summary of the results of the safety evaluations of sulfur-containing heterocyclic compounds used as flavouring agents[a,b,c]

Flavouring agent	No.	CAS no. and structure	*Step B3*[d] Does estimated dietary exposure exceed the threshold of concern?	*Step B4*[e] Adequate margin of exposure for the flavouring agent or a related substance?	Comments on predicted metabolism	Related structure name (No.) and structure (if applicable)	Conclusion based on current estimated dietary exposure
Structural class II							
Triethylthialdine	2205	54717-17-8 	No, SPET: 5	Yes. The NOAEL of 9.3 mg/kg bw per day for the structurally related 5,6-dihydro-2,4,6-tris-(2-methylpropyl)-4*H*-1,3,5-dithiazine (No. 1048) in a 90-day study in rats is 93 000 times the estimated dietary exposure to No. 2205 when used as a flavouring agent.	Notes 1 and 2	5,6-Dihydro-2,4,6-tris(2-methylpropyl)-4*H*-1,3,5-dithiazine (No. 1048) 	No safety concern
Structural class III							
2-Isopropyl-4-methyl-3-thiazoline	2206	67936-13-4 	No, SPET: 75	Yes. The NOAEL of 1.2 mg/kg bw per day in a 90-day study in rats for the structurally related 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) is 1200 times the estimated dietary exposure to No. 2206 when used as a flavouring agent.	Notes 1 and 2	2-(2-Butyl)-4,5-dimethyl-3-thiazoline (No. 1059) 	No safety concern

Flavouring agent	No.	CAS no. and structure	Conclusion
Flavouring agent not evaluated according to the Procedure			
3-Acetyl-2,5-dimethylthiophene	1051	2530-10-1 	Inappropriate for use as a flavouring agent

[a] Fifty-nine flavouring agents in this group were previously evaluated by the Committee (Annex 1, references *161*, *188* and *212*). At the current meeting, No. 1051 was reconsidered in light of potential genotoxicity concerns.

[b] *Step 1*: One flavouring agent is in structural class II (No. 2205), and one flavouring agent is in structural class III (No. 2206).

[c] *Step 2*: The two flavouring agents in this group cannot be predicted to be metabolized to innocuous products.

[d] The thresholds for human dietary exposure for structural classes II and III are 540 µg/day and 90 µg/day, respectively. All dietary exposure values are expressed in µg/day. The dietary exposure values listed represent the highest daily dietary exposures calculated using either the SPET or the MSDI method. The SPET gave the highest estimated dietary exposure in each case.

[e] The MOEs were calculated based on the estimated dietary exposure calculated by the SPET.

Notes:

1. Flavin-dependent monooxygenase and cytochrome P450–dependent sulfoxidation and sulfone formation.
2. Cytochrome P450–dependent alkyl side-chain oxidation.

and *212*). Generally, dithiazine and thiazoline derivatives, being cyclic sulfides, are metabolized primarily by *S*-oxidation to yield the corresponding sulfoxides and sulfones. Other routes of metabolism for sulfur-containing heterocyclic compounds, including ring oxidation and cleavage, are also possible.

Genotoxicity

At the current meeting, the Committee re-evaluated No. 1051 due to concerns about potential mutagenicity. The Committee decided that the positive in vitro and in vivo mutagenicity data suggest a genotoxic risk to humans. Therefore, the Committee considered that it could not evaluate this flavouring agent according to the Procedure (see Table 8).

Application of the Procedure for the Safety Evaluation of Flavouring Agents

The evaluations for Nos 2205 and 2206 were conducted using the Procedure for the Safety Evaluation of Flavouring Agents, as described below.

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents to the above-mentioned flavouring agents, the Committee assigned flavouring agent No. 2205 to structural class II and flavouring agent No. 2206 to structural class III *(6)*.

Step 2. Neither of the flavouring agents in this group (Nos 2205 and 2206) can be predicted to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the B-side of the Procedure.

Step B3. The highest estimated dietary exposure for the flavouring agent in structural class II (No. 2205) is below the threshold of concern (i.e. 540 µg/day for class II). The highest estimated dietary exposure for the flavouring agent in structural class III (No. 2206) is also below the threshold of concern (i.e. 90 µg/day for class III). Accordingly, the evaluation of these flavouring agents proceeded to step B4.

Step B4. For triethylthialdine (No. 2205), the NOAEL of 9.3 mg/kg bw per day for the structurally related 5,6-dihydro-2,4,6-tris(2-methylpropyl)-4*H*-1,3,5-dithiazine (No. 1048) in a 90-day study in rats provides an MOE of 93 000 in relation to the highest estimated dietary exposure to No. 2205 (SPET = 5 µg/day or 0.1 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded that triethylthialdine (No. 2205) would not pose a safety concern at current estimated dietary exposures.

For 2-isopropyl-4-methyl-3-thiazoline (No. 2206), the NOAEL of 1.2 mg/kg bw per day for the structurally related 2-(2-butyl)-4,5-dimethyl-3-thiazoline (No. 1059) in a 90-day study in rats provides an MOE of 1200 in relation to the highest estimated dietary exposure to No. 2206 (SPET = 75 µg/day or 1 µg/kg bw per day) when used as a flavouring agent. The Committee therefore concluded

that 2-isopropyl-4-methyl-3-thiazoline (No. 2206) would not pose a safety concern at current estimated dietary exposures.

Table 8 summarizes the evaluations of the two additional flavouring agents belonging to the group of sulfur-containing heterocyclic compounds (Nos 2205 and 2206).

Consideration of combined intakes from use as flavouring agents

The two additional flavouring agents in this group of sulfur-containing heterocyclic compounds have low MSDI values (0.01 μg/day). The Committee concluded that consideration of combined intakes is not necessary, because the additional flavouring agents would not contribute significantly to the combined intake of this flavouring group.

Conclusion

In the previous evaluations of flavouring agents in this group of sulfur-containing heterocyclic compounds, studies of acute toxicity, short-term toxicity and genotoxicity were available (Annex 1, references *161*, *188* and *212*). None of the flavouring agents in this group raised safety concerns in the previous evaluations.

For the present evaluation, additional short-term studies of toxicity were available for two flavouring agents previously evaluated in this group (Nos 1048 and 2106); studies of in vitro genotoxicity were available for six flavouring agents previously evaluated in this group (Nos 1038, 1045, 1050, 1051, 1059 and 1759), and studies of in vivo genotoxicity were available for two flavouring agents previously evaluated in this group (Nos 1050 and 1051). For one compound (No. 1051) in this group, recently conducted mutagenicity studies suggest a potential mutagenic risk of the substance itself or a reactive metabolite.

The Committee concluded that the new data available indicate that 3-acetyl-2,5-dimethylthiophene (No. 1051) is mutagenic in vitro and in vivo; although the mechanism of mutagenesis is unknown, the possibility of a mutagenic response in humans cannot be discounted. Additional toxicity and metabolic studies that would have been relevant in assessing the biological significance of the mutagenicity evidence reported in in vitro and in vivo assays were not available. The Committee considered it inappropriate for such a compound to be used as a flavouring agent or for any other food additive purpose and withdrew the previous conclusion of the Committee. The Committee is also aware that the flavouring industry has already taken steps to remove this compound from the market.

The Committee concluded that the flavouring agents Nos 2205 and 2206, which are additions to the group of sulfur-containing heterocyclic compounds evaluated previously, would not give rise to safety concerns at current estimated dietary exposures.

An addendum to the toxicological monograph was prepared.

4.2 Specifications of identity and purity of flavouring agents

The Committee received information related to specifications for the 26 new flavouring agents from the call for data for the present meeting. At the current meeting, no specifications were prepared for α-ionene (No. 2193) because it was not evaluated toxicologically (see section 4.1.1) or for (±)-2-phenyl-4-methyl-2-hexenal (No. 2069) due to unresolved toxicological concerns (see section 4.1.7). Specifications were prepared for 24 flavouring agents. In addition to the free acid form of No. 2204, the Committee was notified that the hemisulfate monohydrate salt of this flavouring was also used in commerce. As a result, specifications were also prepared for the hemisulfate monohydrate salt of No. 2204, and this substance was identified as No. 2204.1.

Specifications established at the seventy-sixth meeting in 2012 (Annex 1, reference *211*) for nerolidol oxide (No. 2137) were maintained.

Specifications established at the fifty-ninth meeting in 2002 (Annex 1, reference *160*) for 3-acetyl-2,5-dimethylthiophene (No. 1051) were withdrawn based on toxicological concerns (see section 4.1.8).

5. Future work and recommendations

General considerations

Threshold of toxicological concern (TTC) principle: update on a WHO project and implications for the Procedure for the Safety Evaluation of Flavouring Agents

The Committee recommended that a proposal regarding a revised JECFA decision-tree for the evaluation of flavours based on application of the TTC principle in the risk assessment of chemicals should be further considered and a proposal prepared for consideration at the next JECFA meeting at which flavours will be evaluated.

Need for an approach for prioritizing flavouring agents for re-evaluation

The Committee held a preliminary discussion concerning the fact that the submission of additional toxicology data, including genotoxicity data, and/or exposure data for new or previously evaluated flavouring agents may trigger the need for re-evaluation of previously evaluated flavouring agents. The Committee recommended that an approach be developed for prioritizing flavouring agents for re-evaluation based on all available toxicological data and updated exposure estimates.

Limits for lead in specifications of food additives for use in infant formulas

The Committee referred back to CCFA on whether specific purity criteria for additives for use in infant formulas should be considered and appropriate ways to present these criteria (e.g. establishing specifications for additives for use in infant formulas only; establishing different purity limits for additives for use in infant formulas in existing specifications).

Specific food additives (other than flavouring agents)

Citric and fatty acid esters of glycerol (CITREM)

The Committee noted that the test method for the determination of total citric acid in the specifications monograph for CITREM currently employs a gas chromatographic method using a packed column. The Committee recommended the submission of data for a suitable method using a capillary/wide-bore column to replace the current method for consideration at a future meeting.

Gardenia yellow

The Committee noted that it is not clear whether the material tested toxicologically was representative of gardenia yellow. In addition, the available toxicity studies have

not been conducted following internationally recognized guidelines, and a number of studies were performed using non-relevant routes of administration. Finally, there are no long-term toxicity, carcinogenicity, reproductive toxicity or developmental toxicity studies available.

In order to establish specifications, the Committee requires:

- information on the manufacturing process, including purification steps;
- analytical data on the composition of the substance, including the total amount of colouring matter and relevant compounds of known biological activity, such as geniposide and genipin;
- data on loss on drying;
- information on a method of assay;
- analytical data on at least five different batches of commercial materials supporting the specifications; and
- data on stability in food.

Lutein esters from *Tagetes erecta*

New tentative specifications were prepared. The Committee requested the following information, **by the end of 2015**, to complete the safety assessment:

- details on the manufacturing process, including purification steps;
- detailed analytical data on the full composition of at least five different batches of commercially available product to support the specifications;
- method of analysis to determine carotenoid composition; and
- method of analysis to determine the composition of the non-carotenoid lipidic fraction.

Octenyl succinic acid (OSA)–modified gum arabic

The existing specifications were revised and their tentative status was maintained, pending the submission of the following information, **by the end of 2015**:

- data on the manufacturing process, including purification steps;
- chemical characterization of the product in commerce;
- updated analytical methods for the determination of esterified (bound) and residual (free) OSA;
- results of the analysis of at least five batches of product in commerce; and
- applicability of the high-performance liquid chromatographic method for the determination of residual OSA.

Modified starches

The existing specifications monograph for modified starches includes 16 different modified starches, which complicates revisions of the specifications for any individual modified starch. Therefore, the Committee recommended that the specifications monograph for the modified starches be split into 16 individual specifications monographs.

The Committee, as noted at its seventy-sixth meeting, considered that it would also be necessary to revise the specifications for all the modified starches, including test methods, at future meetings.

Pectin

The Committee requested additional data to support the safety evaluation of pectin in infant formula, including an explanation for the decreased feed intake and body weight gain in neonatal pigs.

Flavouring agents

Phenyl-substituted aliphatic alcohols and related aldehydes and esters

The Committee concluded that the Procedure for the Safety Evaluation of Flavouring Agents could not be applied to (±)-2-phenyl-4-methyl-2-hexenal (No. 2069) until concerns regarding genotoxicity are resolved. In addition, the evaluations of the other α,β-unsaturated aldehydes in this group (Nos 1472–1494 and 1476) should be reconsidered at a future meeting, given the potential genotoxicity of 2-phenyl-2-butenal (No. 1474).

Additional data required to complete the evaluation according to the Procedure for the Safety Evaluation of Flavouring Agents

Additional toxicological and/or dietary exposure information is required to complete the toxicological evaluation of one flavouring agent (No. 2188). The Committee was aware of additional genotoxicity data reporting equivocal results for a structurally related compound; therefore, information to address any concerns regarding potential genotoxicity should also be provided.

Acknowledgements

The Committee wishes to thank Ms M. Sheffer, Ottawa, Canada, for her assistance in the preparation of the report. The Committee is also grateful for the presentations by Professor Andrew Renwick on a revised TTC approach under elaboration with possible implications for the JECFA decision-tree for the evaluation of flavouring agents and by Professor Angelo Moretto on a new decision-tree under development by JMPR for the evaluation of pesticide metabolites using the TTC principle.

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Annex 1

Reports and other documents resulting from previous meetings of the Joint FAO/WHO Expert Committee on Food Additives

1. *General principles governing the use of food additives* (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).

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13. *Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances* (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.

14. *Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non-nutritive sweetening agents* (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.

15. *Toxicological evaluation of some flavouring substances and non-nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.

16. *Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents.* FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.

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Annex 2

Toxicological information and information on specifications

Food additives considered for specifications only

Food additive	Specifications
Citric acid	R[a]
Gellan gum	R[b]
Polyoxyethylene (20) sorbitan monostearate	R[c]
Potassium aluminium silicate	R[d]
Quillaia extract (Type 2)	R[e]

R: existing specifications revised

[a] The method for the oxalate limit test was amended.

[b] The method of assay in the specifications refers to the alginates assay method. This method was replaced by a method without the use of mercury.

[c] Criteria for saponification and hydroxyl values were revised.

[d] The Committee reviewed the existing data as well as new information received from the sponsor and noted that potassium aluminium silicate (PAS) stabilizes the formed layers of titanium dioxide and/or iron oxide in the PAS-based pearlescent pigments. Therefore, the Committee concluded that PAS exerts a technological effect in the PAS-based pearlescent pigments; as a result, PAS could not be considered to function as a carrier according to the Codex definition for carrier. Hence, the Committee decided to delete the functional use as carrier in the specifications.

[e] The upper limit in the loss on drying specification was increased from 80% to 90%.

Food additives evaluated toxicologically and assessed for dietary exposure

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological or safety recommendations
Benzoe tonkinensis	R[a]	Given the no-observed-adverse-effect level (NOAEL) of 500 mg/kg body weight (bw) per day for Benzoe tonkinensis identified in a 90-day oral toxicity study in rats and the previously established ADIs for the major components of Benzoe tonkinensis (benzoic acid, benzyl benzoate and vanillin), **the Committee confirmed the conclusion from the seventy-fourth meeting that Benzoe tonkinensis would not be of safety concern at current estimated dietary exposures**, provided that it complies with the specifications prepared at the current meeting, when used as a flavouring agent and in accordance with good manufacturing practice.
Carrageenan (for use in infant formula and formula for special medical purposes intended for infants)	R	The margins of exposure (MOEs) between the NOAEL of 430 mg/kg bw per day (2250 mg/kg formula), the highest dose tested, from a neonatal pig study and human infant exposures at 2–4 weeks of age range from 2 to 12 on a body weight basis and from 2 to 8 on a concentration basis. The Committee noted that although the MOEs are small in magnitude, they are derived from a neonatal pig study in which the highest dose tested was without adverse effects on the gut or on immune parameters, supported by a neonatal minipig study. These new studies allay the earlier concerns that carrageenan, which is unlikely to be absorbed, may have a direct effect on the immature gut. The Committee also took account of the previous toxicological database on carrageenan, which did not indicate other toxicological concerns. It also noted that at carrageenan concentrations higher than 2500 mg/kg, formula becomes highly viscous, which adversely affects palatability and growth.

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological or safety recommendations
		The Committee concluded that the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1000 mg/L is not of concern. The Committee recognized that there is variability in medical conditions among infants requiring formulas for special medical purposes that contain the higher levels of carrageenan, and the Committee noted that these infants would normally be under medical supervision.
Citric and fatty acid esters of glycerol (CITREM) (for use in infant formula and formula for special medical purposes intended for infants)	R	The Committee considered it unlikely that consumption of formulas containing typical levels of CITREM used in powdered formulas (up to 2.7 g/L as reconstituted), which is equivalent to an exposure to citrate of 440 mg/kg bw per day for the very young infant at the 95th percentile energy intake, would cause diarrhoea. At the high end of the requested range for use (up to 9 g/L), which is equivalent to an exposure to citrate of 1140 mg/kg bw per day for the very young infant at the 95th percentile energy intake, diarrhoea might occur in some infants. **The Committee concluded that there are no toxicological concerns about the use of CITREM in infant formula and formula for special medical purposes at concentrations up to 9 g/L.** At the higher use levels, there is a possibility of diarrhoea from free citric acid released from formula containing CITREM. Given the paucity of clinical data and the fact that exposure assumptions for citric acid have been maximized, it is difficult to estimate the risk of diarrhoea, but it is considered to be low.
Gardenia yellow	No[b]	Given the deficiencies in the toxicological and specifications databases, including incomplete data on the manufacturing process and composition of the material, missing toxicological studies (e.g. long-term toxicity, carcinogenicity, reproductive toxicity and developmental toxicity), the inadequate characterization of the test material and limited reporting of the available studies, **the Committee was unable to evaluate gardenia yellow proposed for use as a food colour.**
Lutein esters from *Tagetes erecta*	N, T[c]	The Committee concluded that there was no need to establish a numerical ADI. This decision was based on a number of factors, including the absence of any observed toxicity of lutein or lutein esters in any of the available toxicological studies in animals; the absence of any adverse effects in humans consuming lutein or lutein esters; the large MOE (>1500) between the NOAEL for lutein in a new 13-week study in rats and the estimated dietary exposure of 0.32 mg/kg bw per day (from additive and natural sources); a 2-fold increase in the NOAEL for lutein as a result of another new 13-week study; and the fact that lutein esters from *Tagetes erecta* are considered to be substitutional for other lutein extracts. **The Committee established a temporary ADI "not specified"[e] for lutein esters from *Tagetes erecta*.** The ADI was made temporary because the specifications for lutein esters from *Tagetes erecta* were tentative. The Committee considered establishing a group ADI "not specified" for lutein esters from *Tagetes erecta* that would include lutein from *Tagetes erecta* and synthetic zeaxanthin and related xanthophylls, but this would be possible only when the specifications for lutein esters from *Tagetes erecta* are finalized.

Food additive	Specifications	Acceptable daily intakes (ADIs) and other toxicological or safety recommendations
Octenyl succinic acid (OSA)–modified gum arabic	R, T[c]	The tentative status of the specifications was maintained pending the submission of additional data. The Committee noted that additional safety data may also be needed to complete the evaluation of OSA-modified gum arabic. **The Committee decided that the temporary ADI "not specified" will be withdrawn unless adequate data to complete the safety evaluation are submitted by the end of 2015.**
Octenyl succinic acid (OSA)–modified starch (starch sodium octenyl succinate) (for use in infant formula and formula for special medical purposes intended for infants)	R[d]	Taking into account the overall low toxicity of OSA-modified starch, the conservatism in the NOAEL, which was the highest dose tested in a study in neonatal animals, and in the exposure assessments, as well as the supporting evidence from clinical trials and post-marketing surveillance, **the Committee concluded that the consumption of OSA-modified starch in infant formula or formula for special medical purposes intended for infants is not of concern at use levels up to 20 g/L.** New data available since the twenty-sixth meeting confirm the very low toxicity of OSA-modified starch, and **the Committee confirmed the ADI "not specified" established at that meeting for its use as a food additive for the general population.**
Paprika extract	M	**The Committee established an ADI for paprika extract used as a food colour of 0–1.5[f] mg/kg bw, expressed as total carotenoids**, with the application of an uncertainty factor of 100 to the NOAEL of 153 mg/kg bw per day from a 2-year toxicity and carcinogenicity study in rats. The Committee concluded that dietary exposure to paprika extract used as a food colour does not present a health concern.
Pectin (for use in infant formula and formula for special medical purposes intended for infants)	M	In a 3-week study in neonatal pigs fed pectin-containing milk replacer, the NOAEL was 847 mg/kg bw per day, with decreased feed intake and body weight gain observed at 3013 mg/kg bw per day. Using the NOAEL from this study, the MOEs were estimated to be 0.9 for infants with median energy intake and 0.8 for infants with high (95th percentile) energy intake. The Committee concluded that estimated exposure to pectin from its use in infant formula is in the region of the NOAEL derived from the neonatal pig study and close to the LOAEL based on decreased feed intake and body weight gain. While no overt toxicological effects were observed in the neonatal pigs, decreased food intake and body weight gain would be considered an undesirable effect in human infants. The available clinical studies were mainly conducted with pectin or pectin-derived oligosaccharides at concentrations of 0.2% or less and therefore do not provide support for tolerance and normal growth at the proposed use level. Therefore, **the Committee concluded that the use of pectin in infant formulas at the maximum proposed use level (0.5%) is of concern.**

M: existing specifications maintained; N: new specifications; No: no specifications prepared; R: existing specifications revised; T: tentative specifications

[a] The tentative qualification of the specifications was removed.

[b] No specifications were prepared. Information is required to prepare specifications.

[c] Additional information is required to finalize the specifications (see section 5).

[d] The analytical method for the determination of the octenyl succinyl group in starch sodium octenyl succinate was amended.

[e] ADI "not specified" is used to refer to a food substance of very low toxicity that, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary exposure to the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice

– i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.

[f] The Committee noted that although derived values, such as health-based guidance values, should be rounded to a single significant figure, it decided to use two significant figures in the present case, as the impact of rounding to one significant figure would be more than 30%.

Flavouring agents evaluated by the Procedure for the Safety Evaluation of Flavouring Agents

A. Aliphatic and alicyclic hydrocarbons

The Committee determined that the flavouring agent α-ionene (No. 2193), which was submitted for evaluation as part of this flavouring agent group, did not fit into this group on the basis of its chemical structure and did not evaluate α-ionene.

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
1-Octene	2191	N	No safety concern
2,4-Nonadiene	2192	N	No safety concern
4-Methyl-*cis*-2-pentene	2194	N	No safety concern
1-Nonene	2195	N	No safety concern
1,3,5,7-Undecatetraene	2196	N	No safety concern
Mixture of methyl cyclohexadiene and methylene cyclohexene	2197	N	No safety concern

N: new specifications

B. Aliphatic and aromatic ethers

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class III			
Cassyrane	2189	N	No safety concern
1-Cyclopropanemethyl-4-methoxybenzene	2190	N	No safety concern
Nerolidol oxide	2137	M	No safety concern

M: existing specifications maintained; N: new specifications

C. Ionones and structurally related substances

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
β-Isomethylionone	2186	N	No safety concern
Pseudoionone	2187	N	No safety concern
trans-α-Damascone	2188	N	Additional data required to complete evaluation

N: new specifications

D. Miscellaneous nitrogen-containing substances

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class III			
3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester	2203	N	No safety concern
4-Amino-5-(3-(isopropylamino)-2,2-dimethyl-3-oxopropoxy)-2-methylquinoline-3-carboxylic acid (and its hemisulfate monohydrate salt)	2204 2204.1	N	No safety concern

N: new specifications

E. Monocyclic and bicyclic secondary alcohols, ketones and related esters

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class II			
2,2,6,7-Tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-ol	2198	N	No safety concern
dl-Camphor	2199	N	No safety concern
l-Fenchone	2200	N	No safety concern
2,2,6,7-Tetramethylbicyclo[4.3.0]nona-4,9(1)-dien-8-one	2201	N	No safety concern

N: new specifications

F. Phenol and phenol derivatives

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class II			
Myricitrin	2207	N	No safety concern
Structural class III			
Naringin dihydrochalcone	2208	N	No safety concern
1-(2,4-Dihydroxyphenyl)-3-(3-hydroxy-4-methoxyphenyl)propan-1-one	2209	N	No safety concern
(−)-Matairesinol	2210	N	No safety concern

N: new specifications

G. Phenyl-substituted aliphatic alcohols and related aldehydes and esters

The Committee concluded that the Procedure could not be applied to (±)-2-phenyl-4-methyl-2-hexenal (No. 2069) until concerns regarding genotoxicity are resolved. In addition, the evaluations of the other α,β-unsaturated aldehydes in this group (Nos 1472–1494 and 1476) should be reconsidered at a future meeting, given the potential genotoxicity of 2-phenyl-2-butenal (No. 1474).

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class I			
Ethyl 3-(2-hydroxyphenyl)propanoate	2202	N	No safety concern

N: new specifications

H. Sulfur-containing heterocyclic compounds

The Committee concluded that 2,5-dimethyl-3-acetylthiophene (No. 1051) is mutagenic in vitro and in vivo and considered that it is inappropriate for such a compound to be used as a flavouring agent or for any other food additive purpose. It therefore withdrew the previous conclusion of the Committee. The Committee is also aware that the flavouring industry has already taken steps to remove this compound from the market. Specifications established at the fifty-ninth meeting for No. 1051 were also withdrawn based on toxicological concerns.

Flavouring agent	No.	Specifications	Conclusion based on current estimated dietary exposure
Structural class II			
Triethylthialdine	2205	N	No safety concern
Structural class III			
2-Isopropyl-4-methyl-3-thiazoline	2206	N	No safety concern

N: new specifications

Annex 3

Summary of the safety evaluation of the secondary components for flavouring agents with minimum assay values of less than 95%

JECFA No.	Flavouring agent	Minimum assay value	Secondary components	Comments on secondary components
Aliphatic and alicyclic hydrocarbons				
2192	2,4-Nonadiene	79–80%	10–11% 1,3-nonadiene; 9–10% other nonadiene isomers	1,3-Nonadiene and other nonadiene isomers are anticipated to undergo oxidative metabolism to the corresponding epoxide followed by hydrolysis by epoxide hydrolase and glucuronic acid conjugation and elimination in the urine

Annex 4

Meeting agenda

Food and Agriculture Organization of the United Nations

World Health Organization

79th JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES (JECFA)
WHO Headquarters, Geneva, 17–26 June 2014

Opening:
Salle B, 17 June 2014, 9.30h

Agenda

1. Opening

2. Election of Chairperson and Vice-Chairperson, appointment of Rapporteurs

3. Adoption of Agenda

4. Declarations of Interests (information by the Secretariat on any declared interests and discussion)

5. Matters of interest arising from previous Sessions of the Codex Committee on Food Additives
   a. Report from CCFA - questions for action

6. Critical issues and questions from Working Papers (first brief round of discussion on all subjects to inform the full committee)

7. Evaluations

   Food Additives

   7.1 Toxicological evaluation, exposure assessment, and establishment of specifications:
   - Carrageenan (INS 407)
   - Citric acid esters of mono- and diglycerides of fatty acids (CITREM) (INS 472c)
   - Gardenia yellow (crocin)
   - OSA-modified starch (starch sodium octenyl succinate) (INS 1450)
   - OSA-modified gum arabic

- Paprika extract
- Pectin (INS 440)
- *Tagetes* extract (INS 161b(ii))

7.2 Food additives for revision of specifications only:
- Benzoe tonkinesis
- Citric acid
- Gellan gum (INS 418)
- Polyoxyethylene (20) sorbitan monostearate (polysorbate 60) (INS 435)
- *Quillaia* extract, type 2 (INS 999(ii))

Flavourings

7.3 New compounds as additions to previously evaluated groups of related flavouring substances:
- Ionones and structurally related substances
- Aliphatic and aromatic ethers
- Aliphatic and alicyclic hydrocarbons
- Monocyclic and bicyclic secondary alcohols, ketones, and related esters
- Phenyl-substituted aliphatic alcohols and related aldehydes and esters
- Miscellaneous nitrogen-containing substances
- Sulfur-containing heterocyclic compounds
- Phenol and phenol derivatives

8. Other matters to be considered (general considerations):
   a. For information:
      i. Update on GEMS/Food cluster diets
      ii. FOSCOLLAB
      iii. New FOS website
   b. For discussion:
      i. Threshold of toxicological concern principle: update on WHO project and implication for decision-tree for the evaluation of flavours
      ii. Limits for lead in specifications of food additives for infant foods

9. Other matters as may be brought forth by the Committee during discussions at the meeting.

10. Adoption of the report.

SELECTED WHO PUBLICATIONS OF RELATED INTEREST

Evaluation of Certain Veterinary Drug Residues in Food
Seventy-eighth Report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 988, 2014 (127 pages)

Toxicological Evaluation of Certain Veterinary Drug Residues in Food
Seventy-eighth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)
WHO Food Additives Series, No. 69, 2014 (241 pages)

Evaluation of Certain Food Additives and Contaminants
Seventy-seventh Report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 983, 2013 (75 pages)

Safety Evaluation of Certain Food Additives and Contaminants
Seventy-seventh Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)
WHO Food Additives Series, No. 68, 2013 (335 pages)

Evaluation of Certain Food Additives
Seventy-sixth Report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 974, 2012 (190 pages)

Safety Evaluation of Certain Food Additives
Seventy-sixth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)
WHO Food Additives Series, No. 67, 2012 (335 pages)

Evaluation of Certain Veterinary Drug Residues in Food
Seventy-fifth Report of the Joint FAO/WHO Expert Committee on Food Additives
WHO Technical Report Series, No. 969, 2012 (108 pages)

Toxicological Evaluation of Certain Veterinary Drug Residues in Food
Seventy-fifth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)
WHO Food Additives Series, No. 66, 2012 (183 pages)

Safety Evaluation of Certain Food Additives and Contaminants
Seventy-fourth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)
WHO Food Additives Series, No. 65, 2012 (825 pages)

Further information on these and other WHO publications can be obtained from
WHO Press, World Health Organization ▪ 1211 Geneva 27, Switzerland ▪ www.who.int/bookorders
tel.: +41 22 791 3264; **fax:** +41 22 791 4857; **email:** bookorders@who.int

Evaluation of certain food additives

This report represents the conclusions of a Joint FAO/WHO Expert Committee convened to evaluate the safety of various food additives, including flavouring agents, and to prepare specifications for identity and purity.

The first part of the report contains a general discussion of the principles governing the toxicological evaluation of and assessment of dietary exposure to food additives, including flavouring agents. A summary follows of the Committee's evaluations of technical, toxicological and dietary exposure data for eight food additives (Benzoe tonkinensis; carrageenan; citric and fatty acid esters of glycerol; gardenia yellow; lutein esters from *Tagetes erecta*; octenyl succinic acid–modified gum arabic; octenyl succinic acid–modified starch; paprika extract; and pectin) and eight groups of flavouring agents (aliphatic and alicyclic hydrocarbons; aliphatic and aromatic ethers; ionones and structurally related substances; miscellaneous nitrogen-containing substances; monocyclic and bicyclic secondary alcohols, ketones and related esters; phenol and phenol derivatives; phenyl-substituted aliphatic alcohols and related aldehydes and esters; and sulfur-containing heterocyclic compounds).

Specifications for the following food additives were revised: citric acid; gellan gum; polyoxyethylene (20) sorbitan monostearate; potassium aluminium silicate; and *Quillaia* extract (Type 2).

Annexed to the report are tables summarizing the Committee's recommendations for dietary exposures to and toxicological evaluations of all of the food additives and flavouring agents considered at this meeting.

ISBN 978 92 4 120990 8



CARRAGEENAN

VOL.: 31 (1983) (p. 79)

CAS No.: 9000-07-1

5. Summary of Data Reported and Evaluation

5.1 Experimental data

Native (undegraded) carrageenan was tested for carcinogenicity in rats and hamsters by administration in the diet; no evidence of carcinogenicity was found. In female rats treated with azoxymethane or *N*-nitrosomethylurea together with native carrageenan in the diet, a greater incidence of colorectal cancers was observed than with treatment by azoxymethane or *N*-nitrosomethylurea alone. Degraded carrageenan was tested in rats by administration in the diet, in the drinking-water and by oral intubation, in four experiments; colorectal cancers were induced in each study.

Native carrageenan has not been tested in short-term assays. Degraded carrageenan was not mutagenic in bacteria or in mammalian cells *in vitro*. The data were *inadequate* to evaluate the activity of degraded carrageenan in short-term tests.

Native or degraded carrageenan had no reproductive or teratogenic effect in rats or hamsters.

5.2 Human data

Carrageenan-containing seeweeds have been used since at least two hundred years, and isolated carrageenan has been used as a food additive since 1937. Carrageenan is used in the manufacture of food, drug and cosmetic products and in multiple industrial applications, resulting in wide human exposure.

No data were available to assess the teratogenicity or chromosomal effects of this compound in humans.

No case report or epidemiological study on the carcinogenicity of native or degraded carrageenan was available to the Working Group.

5.3 Evaluation

The available data do not provide evidence that native (undegraded) carrageenan is carcinogenic to experimental animals. In the absence of epidemiological data, no evaluation of the carcinogenicity of native carrageenan to humans could be made.

Experiments in rats with doses of degraded carrageenan comparable to those used to test native carrageenan provide *sufficient evidence* for the carcinogenicity of degraded carrageenan in rats. No data on humans were available.

For definition of the italicized terms, see Preamble Evaluation.

Previous evaluation: Vol. 10 (1976)

Subsequent evaluation: Suppl. 7 (1987) (p. 59: Carrageenan, native - **Group 3**; Carrageenan, degraded - **Group 2B**)

Synonyms

- Aubygel GS, LGA, MR5O, TRS, X52, X100 & X120
- Aubygum DM
- Burtonite V-40-E
- Carastay
- Carastay C, E, K, M, S, & X; Colloid 775
- Carrageen
- κ, λ and ι-Carrageenan
- Carrageenan gum
- Carrageenin
- Carragheanin
- Carragheen
- Carragheenan
- Chondrus extract
- Coreine
- Eucheuma spinosum gum
- Flanogen ELA, RS1; RS2, 531 & 553
- Galozone
- Gelcarin
- Gelcarin HMR, LA, SI
- Gelogen 2, 4, 8, P10, 28, 406 & 440
- Gelozone
- Genu
- Genugel
- Genugel CJ, CMJ-2, CMJ-343, CWG, CWG-122, KWG, LC-1, LC-4,MGW, PWG, UE & WG
- Genugol RLV
- Genulacta
- Genulacta CL-126, CP-100, K-100, L-100, MDS, P-100 & PL-93
- Genuvisco J
- Gum carrageenan
- Gum chon 2
- Gum chond
- Irish gum

- Irish moss extract
- Lygomme CDS, DP, GB3, LA60, 34, 35 & 267/3
- Pellugel
- Pellugel ID
- Pencogel
- Satiagel GS350, HV, HVX, K40 & K80
- Satiagum 3
- Satiagum standard
- Seakem Carrageenin
- Seakem 3 & LCM
- Seaspen PF
- Sulphate ester of a polysaccharide of galactose
- Viscarin
- Viscarin 402 & TP-4

Last updated: 16 April 1998

Safety Assessment of Polysaccharide Gums as Used in Cosmetics

Status: Final Report
Release Date: October 22, 2015
Panel Date: September 21-22, 2015

The 2015 Cosmetic Ingredient Review Expert Panel members are: Chair, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; Ronald A. Hill, Ph.D.; Curtis D. Klaassen, Ph.D.; Daniel C. Liebler, Ph.D.; James G. Marks, Jr., M.D.; Ronald C. Shank, Ph.D.; Thomas J. Slaga, Ph.D.; and Paul W. Snyder, D.V.M., Ph.D. The CIR Director is Lillian J. Gill, D.P.A. This report was prepared by Wilbur Johnson, Jr., M.S., Senior Scientific Analyst and Bart Heldreth, Ph.D., Chemist.

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ABSTRACT

The Cosmetic Ingredient Review (CIR) Expert Panel (the Panel) reviewed the safety of 106 ingredients, which function as viscosity increasing agents in cosmetic products. The Panel reviewed relevant animal and human data on these ingredients. The Panel concluded that most of the polysaccharide gums are safe in the present practices of use and concentration in cosmetics, as described in this safety assessment, but that the available data are insufficient to make a

determination that hydrolyzed carrageenan is safe under the intended conditions of use in cosmetics. The Panel was concerned about the presence of alkylating and other agents that are used to modify polysaccharide gums in cosmetics. Industry should use good manufacturing practices to limit impurities.

INTRODUCTION

The safety of 106 polysaccharide gums (see Tables 1 and 2) as used in cosmetics is reviewed in this safety assessment. The polysaccharide gums are each naturally derived materials that comprise polysaccharides obtained from plants or algae. Based on the different chemical structures that are associated with polysaccharide gums, these ingredients can be subdivided into categories such as modified, unmodified, linear, branched, and cyclic. Regardless of how they are structured, all of the "moieties" that comprise the molecular structures of these ingredients are polymers composed of monosaccharides.

Although these ingredients could be categorized in multiple ways, all of these ingredients fall into two predominate categories, modified and unmodified. The ingredients in the Modified subgroup have been further subdivided into Linear, Branched, Cyclic, and Unknown Structural Configuration. The ingredients in the Unmodified subgroup have been subdivided into Linear Polysaccharides and Their Salts, Branched - Unmodified, Cyclic, and Unknown Structural Configuration.

Based on chemical similarities, relevant data on the following are included for use in evaluating the safety of ingredients in this review: wheat bran extract (contains ~ 80% arabinoxylan oligopeptides) - for use in the safety assessment of arabinoxylan (branched - unmodified subgroup); pectin-derived acidic oligosaccharides (mixture of linear oligomers and small polymers of galacturonic acid) - for safety assessment of pectin (branched - unmodified subgroup), which consists chiefly of partially methoxylated polygalacturonic acids; and carboxymethyl inulin - for safety assessment of sodium carboxymethyl inulin (branched - modified subgroup). Many of the polysaccharide gums reviewed in this safety assessment function as viscosity increasing agents in cosmetic products.[1] Other functions are listed in Table 2.

As a group, polysaccharide gums comprise polymers of simple saccharide monomers. Their substantial molecular sizes suggest that skin penetration of these ingredients would be unlikely. Thus, these ingredients are unlikely to have significant systemic accessibility and any major decomposition products are likely to be simple saccharides.

In addition, the Panel has issued "safe as used" conclusions for the following cosmetic ingredients which are structurally similar to some of the ingredients reviewed in this safety assessment: galactomannans,[2] microbial polysaccharide gums,[3] astragalus gummifer gum,[4,5] aloe barbadensis leaf polysaccharides,[6] oryza sativa (rice) starch,[7] zea mays (corn) starch,[8] acacia senegal gum,[9] glyceryl alginate,[10] hyaluronic acid,[11] and triticum vulgare (wheat) starch.[12,13]

CHEMISTRY

Definition and Structure

Polysaccharide nomenclature follows the general principles of established organic and carbohydrate nomenclature. Polysaccharide (glycan) is the name given to a macromolecule consisting of a large number of monosaccharide (glycose) residues joined to each other by glycosidic linkages (Figure 1). The term poly(glycose) is not a synonym for polysaccharide (glycan), because it refers to macromolecules composed of glycose residues joined to each other by non-glycosidic linkages. Polysaccharides may be linear, branched, or cyclic. Definitions, structures, and functions of the polysaccharide gums reviewed in this safety assessment, as used in cosmetics and defined in the *International Cosmetic Ingredients Dictionary and Handbook*, are presented in Tables 1 and 2.[1]

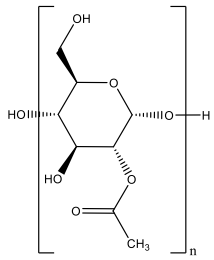


Figure 1. Starch Acetate – an example of a polysaccharide gum

The polysaccharide gums are each naturally derived materials that comprise polysaccharides obtained from plants or algae. Their substantial molecular sizes suggest that skin penetration of these ingredients would be unlikely. While, for the sake of clarity and organization, these ingredients can be subdivided into categories such as linear, branched, cylic, modified, and unmodified, these moieties represent a family of structurally similar polymeric materials, composed of simple saccharide monomers. So, in intended cosmetic application, these ingredients are unlikely to have significant systemic accessibility and any major decomposition products are likely to be simple saccharides, albeit chemically modified ones in some instances (*vide supra*).

Physical and Chemical Properties

Physical and chemical properties of polysaccharide gums are presented in Table 3. These gums have high molecular weights, and many are insoluble in water.

Method of Manufacture

Methods of manufacture of polysaccharide gums are presented in Table 3. The manufacturing processes for hydrolyzed furcellaran and starch hydroxypropyltrimonium chloride are presented in the following sections.

Linear – Modified

Hydrolyzed Furcellaran

The manufacturing process for hydrolyzed furcellaran is presented in Figure 2 below.

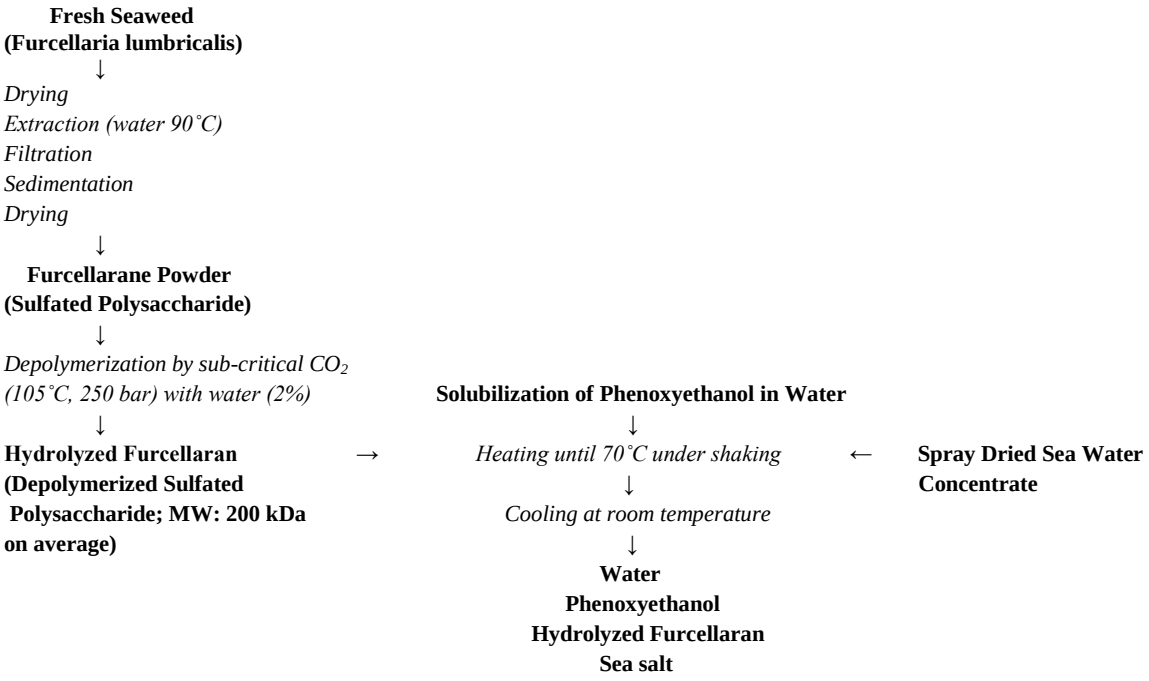


Figure 2. Manufacturing Process for Hydrolyzed Furcellaran.[14]

Branched – Modified

Starch Hydroxypropyltrimonium Chloride

The manufacturing process for starch hydroxypropyltrimonium chloride is presented in Figure 3 below.

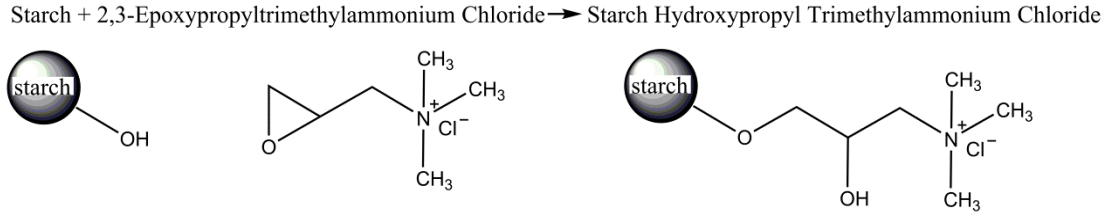


Figure 3. Reaction to form cationic starch ether.[15]

Composition/Impurities

Composition and impurities data on polysaccharide gums are presented in Table 4. Composition/properties data on two hydrolyzed starch products are presented in Table 5.

USE

Cosmetic

Many of the ingredients reviewed in this safety assessment function as viscosity increasing agents in cosmetic products, and the complete list of polysaccharide gum functions in cosmetic products is presented in Table 2.[1] According to information supplied to the Food and Drug Administration (FDA) by industry as part of the Voluntary Cosmetic Registration Program (VCRP), and the results from a survey of ingredient use concentrations conducted by the Personal Care Products Council (Council) in 2013, 58 of these polysaccharide gums are being used in cosmetic products and maltodextrin has the highest reported use frequency.[16,17,18,19]

The Council survey data also indicate that polysaccharide gums are being used in rinse-off cosmetic products at maximum ingredient use concentrations up to 50% (i.e., for algin in paste masks and mud packs), and in leave-on cosmetic products at maximum ingredient use concentrations up to 45.7% (i.e., for corn starch modified in tonics, dressings, and other hair grooming aids).[16,18] Frequency of use/use concentration data for polysaccharide gums are summarized in Table 6.

Cosmetic products containing polysaccharide gums may be applied to the skin and hair or, incidentally, may come in contact with the eyes (maximum ingredient use concentration in these products = 30%) and mucous membranes (maximum ingredient use concentration in these products = 32%). Products containing these ingredients may be applied as frequently as several times per day and may come in contact with the skin or hair for variable periods following application. Daily or occasional use may extend over many years.

Polysaccharide gums are used at concentrations up to 9.5% (avena sativa (oat) starch) in cosmetic products that are sprayed, which also includes use in a pump hair spray at a maximum concentration of 0.45% (corn starch modified), and at concentrations up to 45.7% (corn starch modified) in cosmetic products that possibly are sprayed. Ingredient use in underarm aerosol deodorant sprays is being reported at maximum use concentrations ranging from 0.001% (algin) to 2.5% (cyclodextrin). Hydroxypropyl cyclodextrin is being used in underarm pump deodorant sprays at a maximum use concentration of 0.34%. Additionally, polysaccharide gums are used in powders at concentrations up to 33% (tapioca starch). Because polysaccharide gums are used in products that are sprayed, they could possibly be inhaled. In practice, 95% to 99% of the droplets/particles released from cosmetic sprays have aerodynamic equivalent diameters >10 µm, with propellant sprays yielding a greater fraction of droplets/particles below 10 µm, compared with pump sprays.[20,21,22,23] Therefore, most droplets/particles incidentally inhaled from cosmetic sprays would be deposited in the nasopharyngeal and bronchial regions and would not be respirable (i.e., they would not enter the lungs) to any appreciable amount.[20,21] There is some evidence indicating that deodorant spray products can release substantially larger fractions of particulates having aero-dynamic equivalent diameters in the range considered to be respirable.[21] However, the information is not sufficient to determine whether significantly greater lung exposures result from the use of deodorant sprays, compared to other cosmetic sprays.

Non-cosmetic

According to the FDA, the following polysaccharide gums are approved direct food additives affirmed as generally recognized as safe (GRAS):[24,25] agar, alginic acid, ammonium alginate, amylose (i.e., high-amylose corn starch is GRAS), calcium alginate, pectin, potassium alginate, dextrin, maltodextrin, solanum tuberosum (potato) starch, solanum tuberosum

(potato) starch, starch acetate, tapioca starch, hydroxypropyl starch, propylene glycol alginate, carrageenan, ghatti gum, and sterculia urens gum.

Linear Polysaccharides and Their Salts

Algin

The viscosity of blood substitutes is among the important determinants in restoring microcirculation.[26] Sodium alginate (algin) is frequently mentioned as a viscosity modifier in the development of blood substitutes.

Alginates

Alginate dressings are among the types of absorbent dressings that are used to treat exuding wounds.[27]

Carrageenan

κ-Carrageenan (thickening agent) stabilizes milk proteins and is widely used in dairy products.[28]

At the June 2014 meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the Committee concluded that the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1000 mg/L is not of concern.[29] Furthermore, the Committee recognized that there is variability in medical conditions among infants requiring formulas for special medical purposes that contain the higher levels of carrageenan, and noted that these infants would normally be under medical supervision. A summary of the discussion on which the Committee's conclusion is based is summarized in the Repeated Dose Toxicity-Oral section of this report.

Inulin

Inulin is a prebiotic, meaning a non-digestible food ingredient that selectively stimulates the growth and/or activity of one or several bacterial species in the colon.[30]

Branched - Unmodified

Ghatti Gum

Ghatti gum (thickening agent) is used to stabilize table syrup emulsions, as a glaze in candy products, and as a component of chewing gum, cough drops, and candy lozenges.[28]

Sterculia Urens Gum

Sterculia urens gum has the following uses in food: formulation aid, stabilizer and thickener, and emulsifier and emulsifier salt.[31] World Health Organization (WHO) reports affirming the safety of karaya gum as a food additive are available.[32,33]

Cyclic

Cyclodextrin

Cyclodextrins have been used to solubilize drugs in aqueous vehicles as guest-host complexes.[34]

TOXICOKINETICS

Non-Human

Linear Polysaccharides and Their Salts

Carrageenan

Carrageenan is not degraded or absorbed in the gastrointestinal tracts of rodents, dogs, and non-human primates.[35]

Branched - Unmodified

Sterculia Urens Gum

A toxicokinetic study on sterculia urens gum was performed using 2 groups of 4 male Sprague-Dawley rats of the CD strain. One group was fed a pelleted diet containing 5% sterculia urens gum for 24 h, and the control group was fed a similar laboratory pelleted diet without the gum. Urine and feces were collected and weighed after 24 h, 48 h, and 72 h. The polysaccharide of sterculia urens gum is composed essentially of rhamnose, galactose and galacturonic acid. Fecal polysaccharide was calculated as sterculia urens gum polysaccharide after correction for background levels of rhamnose, galactose, and galacturonic acid in the control feces. The quantity and monosaccharide composition of the fecal polysaccharide were compared with the dose and original composition of the gum polysaccharide. Aggregated polysaccharide estimated over the 72-h collection period ranged from 81% to 108%, with a mean value of 95% of that consumed. Thus, 95% of the gum ingested was excreted in the feces.[36]

Cyclic

Cyclodextrin

The absorption of orally administered ^{14}C-β-cyclodextrin, in methylcellulose solution, was studied using 4 Wistar R x Long Evans F_1 male rats.[37] Two rats received an oral dose of 36.7 mg/kg, and the other 2 rats received 36.9 mg/kg. The average dose volume was 1.5 ml. The maximum radioactivity of the blood derived from ^{14}C-β-cyclodextrin occurred between the 4th and 11th hour after exposure, and the maximum radioactivity in different experiments ranged from 5% to 17% of the total administered radioactivity. Radioactivity excreted in the urine ranged from 4.2% to 4.8% of the total radioactivity administered. No specific accumulation of ^{14}C-β-cyclodextrin in organs was found after dosing. The large intestine contained 10% to 15% of the ^{14}C-β-cyclodextrin radioactivity at 24 h post-dosing.

In another experiment, a female CFY rat received an oral dose of 313 mg/kg ^{14}C-β-cyclodextrin (homogenized in dextran solution, volume = 2.5 ml). In the 8th hour after dosing, no more than 3 to 50 ppm β-cyclodextrin was detectable in the blood. In a third experiment, a female CFY rat was dosed orally with 36.1 mg/kg ^{14}C-β-cyclodextrin (homogenized in 1 ml dextran solution), and another rat was dosed orally with 313.5 mg/kg ^{14}C-β-cyclodextrin (homogenized in 2.5 ml dextran solution). Three female CFY rats also received an oral dose of 1.88 mg/kg chromatographically purified ^{14}C-β-cyclodextrin (homogenized in 1.5 ml dextran solution). The radioactivity peak was detected in the exhaled air between the 4th to 6th or the 6th to 8th hour, depending on the dose. The total radioactivity exhaled by ^{14}C-β-cyclodextrin-treated rats in 24 h represented 55% to 64% of the administered ^{14}C-β-cyclodextrin. The authors suggested, based on the results of this study, that the rate-determining step in β-cyclodextrin absorption is the enzymatic hydrolysis of β-cyclodextrin to yield linear dextrins, which are rapidly hydrolyzed to maltose and glucose.[37]

Human

Branched - Unmodified

Starch Acetate

The pharmacokinetics of starch acetate (acetyl starch) and hydroxyethyl starch was studied using 2 groups of 16 surgical patients (18 to 70 years old).[38] Patients in one group were initially infused intravenously (i.v.) with 15 mL/kg of a 6% acetyl starch solution, and then up to a maximal dosing volume of 1,000 mL/kg, over a 30-minute period. The other group was infused with a 6% hydroxyethyl starch solution (same dosing volume) according to the same procedure. When compared to hydroxyethyl starch, rapid and nearly complete enzymatic degradation to acetic acid and glucose (and to products that can be excreted renally) was reported for acetyl starch.

Sterculia Urens Gum

Five male volunteers were involved in a study in which 24-h urine samples were collected prior to, and following, the ingestion of 10 g karaya gum for 15 days.[39] Total gum intake was 10-fold greater than the approved average daily intake (ADI) of 0-12.5 mg/kg body weight. The detection limit for rhamnose in the urine was 0.2 µg; however, rhamnose was not detected in any of the urine specimens. The authors noted that if 1% of the rhamnose in 10 g karaya gum appeared in the 24-h urine specimens, it would have been detected. Furthermore, the results of this study confirmed that dietary gum karaya is neither digested nor degraded by enteric bacteria, and is not absorbed to any significant extent in the digestive tract.

Tapioca Starch

Ten men (29 to 41 years old) participated in an oral exposure study.[40] Blood was collected after a 12-h fast. Tapioca starch (30 g) containing 0.1 g aspartame was dissolved in 150 L of water, and the solution or dispersion remained for 3 minutes in boiling water. Subjects then drank the solution 1 to 2 min later. Three tolerance tests were performed, using a crossover design, over three days. Tapioca starch produced a large, rapid increase in plasma glucose concentration, which peaked in 30 minutes and then decreased toward the basal value.

Percutaneous Absorption

Cyclic - Modified

Hydroxypropyl Cyclodextrin

The percutaneous absorption of 2% ^{14}C-2-hydroxypropyl-β-cyclodextrin *in vivo* was studied using 3 to 5 female hairless mice.[41] The test material (100 µL on occlusive patch) was applied to dorsal skin (2 cm^2) for 24 h. Radioactivity in the patches, in the stratum corneum (collected by tape stripping), and in the epidermis and cutis of the skin (obtained by peeling off the treated portion) was measured using a scintillation counter. The percutaneous absorption of ^{14}C-2-hydroxypropyl-β-cyclodextrin through intact skin was extremely low, i.e., ~ 0.02% of the amount applied to the skin. The absorption rate of ^{14}C-2-hydroxypropyl-β-cyclodextrin through skin from which the stratum corneum had been removed by tape stripping was approximately 24% of the amount applied to the skin. The latter finding suggests that the stratum corneum may act as a barrier to the percutaneous absorption of ^{14}C-2-hydroxypropyl-β-cyclodextrin. Thus, the results of this study clearly demonstrate that 2-hydroxypropyl-β-cyclodextrin has low permeability through hairless mouse skin.

TOXICOLOGICAL STUDIES

A toxicity profile of β-cyclodextrin (a cyclic polysaccharide gum) is available from the WHO.[42] The toxicity profile of cyclodextrins can differ depending on the route of administration. For example, β-cyclodextrin administered orally induces limited toxicity.[43,44] In both rats and dogs, β-cyclodextrin is considered to be non-toxic at a daily dose less than 600 mg/kg body weight or at 3% or less in the diet.[45] However, if β-cyclodextrin is administered at higher doses in animals via a subcutaneous (s.c.) route, it will cause a decrease in body weight gain, a decrease in liver weight, and nephrotoxicity, with an increase in kidney weight, proximal tubular nephrosis and cellular vacuolation.[45,46] In another study (rats), s.c. administration of β-cyclodextrin (≥ 450 mg/kg) induced similar changes in kidney proximal tubules.[47] Acute and repeated dose toxicity studies on polysaccharide gums (according to type of exposure) are summarized in Tables 7 and Table 8, respectively. The following acute toxicity studies (according to type of exposure) on polysaccharide gums are summarized in Table 7: inhalation, oral, dermal, intravenous, intrapleural, and transbronchial. Oral and dermal repeated dose toxicity studies on polysaccharide gums are summarized in Table 8.

Cytotoxicity

Linear Polysaccharides and Their Salts

Calcium Alginate

In a cytotoxicity assay, calcium alginate fibers were introduced into human embryonic kidney cells and human fibroblasts.[48] A total of nine experimental groups were prepared according to the following weights of calcium alginate fibers: 0.005, 0.01, 0.02, 0.03, 0.04, 0.05, 0.08, 0.10, and 0.15 g. Next, 1-cm lengths of fibers were cut and sterilized with UV irradiation prior to their addition to the cells. The cells were in their exponential growth phase, and were incubated for 48 h. Calcium alginate fibers were not cytotoxic.

Allergenicity/Immune System Effects

Non-Human

Linear Polysaccharides and Their Salts

Polianthes Tuberosa Polysaccharide

The potential for a modulatory effect on the murine self-defense system by an acidic polysaccharide (ANK-102) produced by *Polianthes tuberosa* cells in liquid culture was examined.[49] The pretreatment (intraperitoneal [i.p.] injection) of C3H/HeN mice with ANK-102 (2 mg in 0.2 ml solution) deteriorated murine survival against lethal infection with *Listeria monocytogenes***,** an intracellular gram positive bacterium eliminated mainly by macrophages through the T-cell mediated immune response. Pretreatment with ANK-102 resulted in the accumulation of Mac 1 and Mac 2 positive cells in the peritoneal cavity of the infected animals and the reduction of Thy 1.2 expression on the surface of the thymocytes. ANK-102 was classified as an immunosuppressive polysaccharide.

Potassium Carrageenan

Male Sprague-Dawley rats (8 animals, 7 weeks old) were injected i.p. with potassium carrageenan (50 mg in 5 ml PBS).[50] The control group received a single injection of PBS (0.5 ml). At 3 weeks post-injection, serum levels of IgM, IgG and slow α_1- and slow α_2-globulins were measured using quantitative radial immunodiffusion (IgG) or immunoelectrophoresis (IgM and slow α-globulins). There was a significant elevation in levels of IgM and slow α_1 globulin that was maximal on day 4; levels returned to normal by day 14. Slow α_2-globulin was detectable within 24 h, reached a peak at day 2, and, in most animals, was no longer measurable by day 14. Levels of IgG were not affected by potassium carrageenan injection.

Branched - Unmodified

Sterculia Urens Gum (a.k.a. Karaya Gum)

The allergenicity of karaya gum was studied in adult male and female guinea pigs (number not stated).[51] Karaya gum (1 g/kg) was dissolved in normal saline to make a 3% solution, which was injected i.p. The gum was also administered orally (1 g/animal daily) for 3 months, or mixed with food (single feeding of 5 g/animal). Egg albumen served as the control in each experiment. Animals that received single i.p. injections or single oral doses were killed at intervals within a range of 4 to 12 weeks after the attempted sensitization. Animals dosed orally daily for 3 months were killed either on the day after the last dose or after an interval of 6 weeks after the last dose. Isolated pieces of small intestine from treated males and females, seminal vesicles from males, and the uterus of females were suspended in an organ bath and exposed to karaya gum or egg albumen for 10 minutes. The organs of animals exposed *in vivo* to karaya gum where challenged first with egg albumen and, later, with karaya gum, and *vice versa*. Study results indicated that allergic sensitivity did not develop in guinea pigs dosed orally (single or repeated doses) or i.p. Injection of albumen resulted in marked allergic sensitization.

An animal model was used to investigate the immunogenicity of karaya gum (*Sterculia* spp.).[52] Groups of [(C57BL/6J x DBA/2)F_1] (BDF_1) mice were intradermally immunized with the gum in Freund's complete adjuvant. Serum antibody levels were measured using an enzyme-linked immunosorbent assay (ELISA), and delayed hypersensitivity responses assayed by a footpad swelling test. Karaya gum elicited systemic immune responses after immunization. Further processing reduced immunogenicity, although there was no evidence that systemic immunity

to complex polysaccharide antigen responses could be completely abolished by processing or purification. Karaya gum caused considerable footpad swelling when injected intradermally.

Human

Branched - Modified

Propylene Glycol Alginate

Following a 7-day control period, 5 male volunteers consumed propylene glycol alginate at a dose of 175 mg/kg body weight for 7 days.[53] This regimen was followed by dosing with 200 mg/kg body weight for an additional 16 days. No allergic responses were reported by, nor observed in, any of the volunteers.

In Vitro

Linear Polysaccharides and Their Salts

Potassium Alginate

The acute tissue reactions to potassium alginate, locally applied to a microvascular bed, were studied using the vital microscopic hamster cheek-pouch model and correlative histology.[54] This experimental model permitted the study of microvascular permeability, blood flow, vessel diameters and leucocyte adhesion to vessel walls intravitally, and leucocyte migration and mast cell degranulation histologically. Deionized water alone and potassium alginate with flavor and color mixed in saline was found to cause severe microvascular alterations, while potassium alginate, without flavor and color, mixed in saline and applied to the microvasculature resulted in a minor inflammatory reaction

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

Reproductive and developmental toxicity data on polysaccharide gums are summarized in Table 9. Except for a dose-dependent increase (40-600 mg/kg) in the incidence of missing skeletal sternebrae in rabbits dosed orally with *kappa/lambda*- carrageenan, the results for polysaccharide gums in reproductive and developmental toxicity studies were essentially negative.

GENOTOXICITY

Genotoxicity data (bacterial and mammalian) on polysaccharide gums are summarized in Table 10. In bacterial assays, the following were not genotoxic either with or without metabolic activation: arabinoxylan, carboxymethyl inulin, carrageenan, ghatti gum, glucomannan, and pectin-derived acidic oligosaccharides. In mammalian assays with and without metabolic activation, wheat bran extract, carboxymethyl inulin, carrageenan, ghatti gum, and glucomannan were not genotoxic. However, results for pectin-derived acidic oligosaccharides in mammalian assays were either equivocal or it was classified as clastogenic, only at highly cytotoxic concentrations. Sterculia urens gum was not genotoxic in cytogenetic assays (*in vitro* and *in vivo*) or in the *in vivo* dominant lethal gene test.

CARCINOGENICITY

Studies relating to the carcinogenicity of polysaccharide gums are summarized in Table 11. Agar (50,000 ppm in diet) was not carcinogenic in rats, and up to 25% sodium alginate in the diet was not carcinogenic in mice. Results relating to the carcinogenic potential of carrageenan were mixed. Carrageenan (25% in the diet) was not carcinogenic in mice, but 15% carrageenan in the diet enhanced the colon tumor incidence in azoxymethane (AOM)- and N-nitrosomethylurea (NMU)-treated rats. In the aberrant crypt focus (ACF) assay, 10% carrageenan in

the diet did not initiate colon tumors, 0.25% carrageenan reduced the number of ACF, and 2.5% carrageenan promoted the growth of ACF in rats. In another study, carrageenan (up to 5% in the diet) did not possess promoting activity for colorectal carcinogenesis in rats. It should also be noted that 5% carrageenan in the diet increased colonic cell proliferation in rats, but that it was concluded that this response was probably adaptive, and would not contribute to the increased risk of colon neoplasia in rats. There was no evidence of carcinogenicity in mice fed 55% starch acetate or in rats fed 5% cyclodextrin in the diet. Pectin (2.5% in diet) caused mucosal hyperplasia of the small intestine of rats. Degraded carrageenan, which may or may not be similar to the cosmetic ingredient hydrolyzed carrageenan, caused colon cancer in rats at dietary concentrations of 5% and 10%, but not 1%, in rats. Degraded carrageenan (also known as poligeenan) results from a manufacturing process of seaweed that involves intentional extensive acid hydrolysis, resulting in sulfated galactose polymers with an average molecular weight of approximately 15,000 Da.[35]

Inulin (15 g in basal diet) inhibited the growth of 2 tumor cell lines that were implanted in mice, and the dietary intake of 4.8% arabinoxylan reduced the occurrence of preneiplastic lesions in rats. Glucomannan (10% in the diet) inhibited the development of spontaneous liver tumors in mice.

IRRITATION AND SENSITIZATION

Dermal Irritation and Sensitization

Skin irritation and sensitization studies on polysaccharide gums are summarized in Table12. The results of animal and human tests indicate that these gums can be mild skin irritants, but are non-sensitizers.

Phototoxicity

Branched - Modified

Sodium Hydrolyzed Potato Starch Dodecenylsuccinate

The phototoxicity of a sodium hydrolyzed potato starch dodecenylsuccinate was evaluated using the *in vitro* neutral red uptake phototoxicity assay.[55] The trade name material (in Hanks' balanced salt solution) was evaluated at concentrations ranging from 68.1 to 1,000 µg/ml in BALB/3T3 clone A31 mouse embryo fibroblast cultures. Chlorpromazine served as the positive control. Following incubation, cultures were irradiated for 50 minutes with 1.7 mW/cm^2 UVA to achieve an irradiated dose of 5 J/m^2. A positive result was defined as a photo-irritant factor (PIF) > 5. The PIF was defined as the EC_{50} without solar simulated light (SSL)/ EC_{50} with SSL. The test material was not considered to have phototototoxicity potential (PIF = 0.8). A PIF of 27.9 was reported for the chlorpromazine positive control.

Clinical Trial

Linear Polysaccharides and Their Salts

Calcium Alginate

Fourteen patients (7 males) with spina bifida were treated for pressure sores. Each patient had calcium alginate dressings applied for 4 to 6 weeks.[56] The mean number of dressings removed per week was 3.5 ± 2.1. Good tolerance to treatment was reported for each patient. It was also noted that no severe side effects were recorded during the trial.

Case Reports

Linear Polysaccharides and Their Salts

Calcium Alginate

A 50-year-old woman was referred for treatment after the discovery of adenoid cystic carcinoma in an excised left submandibular gland.[57] Treatment involved clearing the left submandibular fossa, and selective neck dissections. After removal of the clot (submandibular hematoma), a calcium alginate fiber pack was left in place to control the bleeding. After an extended period, the pack was reported to have stimulated a foreign body reaction which, on a computed tomogram, mimicked a recurrence of the tumor.

Alginate

A 52-year-old general practitioner injected 0.1 ml of an alginate solution into the deep dermis of her left arm.[58] Ten days later, she observed a small pink nodule at the injection site; a bluish papule was observed at 3 months post-injection. A biopsy was performed 2 months after injection. At histopathological examination, a granulomatous reaction involving the deep dermis and the subcutaneous fat was observed. The papule regressed, having resolved completely at 5 months post-injection.

Four of 10 patients injected with an aesthetic injectable resorbable filler consisting of purified alginate (extracted from crusted brown algae), into tear troughs and⁄or dorsa of the hands, developed severe granulomatous reactions within months after injections.[59] The 40% incidence of this disfiguring effect was considered high.

Sodium Carrageenan

Within minutes of receiving a barium enema solution that contained sodium carrageenan, a 26-year-old female had an anaphylactic reaction associated with the following signs/symptoms:[60] abdominal cramps, mild generalized pruritus, generalized urticaria, hypotension, transient loss of consciousness, chest tightness, wheezing, and cyanosis. A skin prick test for a component of the barium enema solution, 0.4% weight/volume sodium carrageenan, were positive (i.e., an 8 mm wheal diameter with surrounding flare). This is the only component of the barium enema solution that yielded a positive reaction.

Ocular Irritation

Non-Human

Linear Polysaccharides and Their Salts

Algin

The ocular irritation potential of algin (2%) was studied in 3 experiments using rabbits (number not stated).[61] Instillation of the test substance was followed by scoring after 1 h, 24 h, 2 days, 3 days, 4 days, and 7 days. Corneal opacity and ulceration or granulation were evaluated. Ocular irritation was graded on a scale of 0 to 110, and an ocular irritation index (OII) was calculated. It was noted that a compound does not provoke any significant injury to the mucous membrane of the eye when no opacity of the cornea occurs and when the ocular irritation index is less than 15. OII values of 3.00, 9.17, and 5.50 were reported in the 3 experiments, respectively. Pathological lesions of the ocular mucosa were not observed.

Carrageenan

Food grade *iota*-carrageenan (one subtype of carrageenan with a specific number and position of sulfate groups on the repeating galactose units) was not irritating to unrinsed eyes of rabbits and was minimally irritating to rinsed eyes.[62]

Branched – Modified

Calcium Starch Isododecenylsuccinate and Corn Starch Modified

A material described as structurally similar to sodium hydrolyzed potato starch dodecenylsuccinate and corn starch modified was evaluated for ocular irritation potential in a study involving 6 New Zealand White

rabbits.[63,64,65] The OECD 405 test protocol was used. The powder (0.1 ml) was placed in one eye of each animal. Iritis was observed in 2 rabbits, and reactions had cleared by day 1. Conjunctival irritation was observed in 6 rabbits, and reactions had cleared by day 3. There was no evidence of corneal opacity or abnormal systemic signs during the observation period. The test material was classified as a minimal ocular irritant.

Corn Starch Modified

Corn starch modified, dry powder form, was placed in one eye of each of 6 New Zealand White rabbits (5 males, 1 female).[66] Iritis was observed in 1 of 6 rabbits, and the reaction had cleared by 24 h post-administration. Mild conjunctival irritation was observed in all 6 rabbits, and reactions had cleared by 48 h post-administration. There was no evidence of corneal opacity or abnormal physical signs in any of the animals tested. The test substance was classified as minimally irritating to the eye.

Dextrin Myristate

The ocular irritation potential of dextrin myristate was studied using 6 New Zealand white rabbits. The test concentration and protocol were not stated. Ocular irritation was not observed.[67]

Dextrin Palmitate

In an ocular irritation study involving 3 New Zealand white rabbits per test substance, dextrin palmitate (concentration and test protocol not stated) did not cause reactions in the cornea or iris. Slight conjunctival redness was observed in one rabbit at 1 h post-instillation, but had resolved after 24 h.[68,69]

Potato Starch Modified

A 16.8% aqueous suspension of potato starch modified was evaluated in an ocular irritation study involving 3 rabbits (strain not stated), according to the OECD 405 test guideline. Conjunctival irritation/edema was observed in the 3 rabbits, and all reactions had cleared in 2 rabbits by 24 h post-instillation. In the remaining rabbit, slight swelling of the conjunctivae remained at 24 h, and the reaction had cleared by 48 h post-instillation. It was concluded that the potato starch modified suspension was slightly irritating to the eyes of rabbits.

The ocular irritation potential of potato starch modified (28-1808) was evaluated according to the OECD 405 protocol using 3 New Zealand White rabbits.[70] An 18.5% solids solution of the test substance (0.1 ml) was instilled into one eye of each animal, and reactions were scored for up to 72 h post-instillation. Abnormal physical signs were not observed during the observation period. Conjunctival irritation was observed in all animals, having cleared by 48 h. Neither corneal opacity nor iritis was observed during the study. Potato starch modified (28-1808) was classified as a minimal ocular irritant.

Stearoyl Inulin

The ocular irritation potential of steraroyl inulin (test concentrations and protocol not stated) was evaluated in two tests, each using 6 Japanese white rabbits. The test substance was classified as practically non-irritating.[71,72]

In Vitro

Linear - Modified

Hydrolyzed Furcellaran

The ocular irritation potential of a trade name mixture containing 1.35% furcellaran powder and 1% phenoxyethanol was evaluated in a cytotoxicity assay involving cultured fibroblasts (source not stated). The method of diffusion on agarose gel was used. The product (pure) was applied to cultures during a 24-h period, and was classified as slightly toxic. This finding was interpreted as almost non-irritating to slightly irritating to the eyes.[73] The ocular irritation potential of another trade name mixture containing 1.35% furcellaran powder, 0.1% potassium sorbate, and 0.05% citric acid was evaluated according to the same procedure, and the same results were reported.[73]

Maltodextrin

The ocular irritation potential of maltodextrin was evaluated using the *in vitro* bovine corneal opacity and permeability assay.[74] In this assay, plastic cassettes mimicking eye structure are used as holders for excised corneas. The posterior chamber was filled with cell support media, and the anterior chamber was filled with an eye gel containing 2.45% maltodextrin. After a 10-minute period, opacity was measured by passing visible light from an opacitometer through the cornea and on to the surface of a light sensor. It was noted that a clear cornea unchanged by the test substance would allow light to pass through and be detected by the sensor. Opaque corneas would produce light scattering (Tyndall effect) and reduced detection that is proportional to the degree of ocular damage. Also, following exposure, fluorescein was added to the anterior chamber of the cassette. The amount of dye passing through the cornea and into the posterior chamber is a measure of corneal permeability, and an increase in corneal permeability is indicative of corneal damage. Based on the results of this study, the eye gel was classified as a non-irritant. The positive control, 5% benzalkonium chloride, was classified as a severe irritant.

In addition, the EPI-Ocular® skin model assay was used to evaluate the ocular irritation potential of an eye gel containing 2.45% maltodextrin.[75] In this assay, the degree of ocular irritation is based on the amount of cytotoxicity observed in tissues exposed to the test substance. Cytotoxicity is measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye. The end point established in this assay is the time required for the test substance to reduce tissue viability by 50% (ET_{50}). An $ET_{50} > 4$ h (non-irritant) was reported for the eye gel. The positive control, Triton X-100, was classified as a mild irritant ($ET_{50} = 28.8$ minutes).

Branched – Modified

Hydroxypropyltrimonium Hydrolyzed Corn Starch

The ocular irritation potential of hydroxypropyltrimonium hydrolyzed corn starch was evaluated using the hen's egg test – utilizing the chorioallantoic membrane (HET-CAM).[76] Fertile White leghorn eggs were used. The chorioallantoic membrane (CAM) of the chick embryo responds to injury with a complete inflammatory reaction that is comparable to that induced in the rabbit ocular irritation test. The test substance (0.3 ml) was administered to the CAM at concentrations of 5%, 10%, and 15%. Results indicated that hydroxypropyltrimonium hydrolyzed corn starch would have practically no irritation potential *in vivo*. It was noted that the CAM results at 5%, 10%, and 15% are equivalent to Draize test results for the test substance at concentrations of 10%, 20%, and 30%.

Mucous Membrane Irritation and Sensitization

Non-Human

Branched - Unmodified

Glucomannan

Konjac flour was evaluated in the following study, but the composition of konjac flour is not stated. However, according to one source, every 100 g of konjac flour contains the following:[77] glucomannan (79.37 mg), protein (1.64 g), fat (0.004 g), phosphorus (57 mg), iron (4.06 mg), zinc (123 mg), manganese (0.2 mg), chromium (0.25 mg), and copper (0.08 mg). Prior to initiation of the study, a sensory irritation study on konjac flour (primary polysaccharide component is glucomannan) was performed using ND4 Swiss Webster mice (number not stated).[78] Sensory irritation was evaluated by monitoring the decrease in respiratory rate during 30 minutes of exposure to konjac flour. The concentration of konjac flour that caused a 50% decrease in the respiratory rate (RD_{50}) was 110 mg/m^3.

A study was performed to investigate whether exposure to food grade konjac flour could produce respiratory hypersensitivity.[78] The composition of the sample tested was in agreement with *Food Chemical Codex* specifications of <8% protein, >75% carbohydrate, and <5% ash. Groups of male Hartley guinea pigs were randomly assigned to the following 4 groups (whole-body exposure in chambers): negative control (4 animals, air-

exposed), positive control (4 animals, trimellitic anhydride [TMA] exposure), and konjac flour exposure group (8 animals). Test animals were exposed to konjac flour on days 1-5 of the study (42 minutes/induction exposure), and challenged (35 minutes/challenge exposure) on days 19, 26, and 40. The mean (± S.D.) konjac flour concentration during induction exposure was 111 ± 8.3 mg/m^3, and the mean exposure concentration during the challenge phase ranged from 50 to 68 mg/m^3. The days of exposure (induction and challenge) for positive control animals exposed to TMA aerosol were identical to those for the test group. The target exposure concentrationof TMA was 94 mg/m^3 for induction and challenge. Negative control animals were exposed to room air on days 1-5, but were challenged with konjac flour (target concentration = 114 mg/m^3) only on day 40 to avoid the possibility of repeated challenges resulting in sensitization.

The criteria used to define respiratory tract sensitization (increase in respiratory rate of 36% and change in respiratory waveform) were achieved in 25% of the animals during each challenge in the konjac flour exposure group. Additionally, a few animals responded with slightly lower increases in respiratory frequency and a change in waveform that were suggestive of a slight pulmonary hypersensitivity response.[78] According to a more recent publication, the purified antigen from konjac flour is named Ag40D-2 (acidic protein; ~ 24,000 daltons), suggesting that the respiratory sensitizer in konjac flour is actually a protein, rather than glucomannan.[79]

Cyclic - Modified

Methyl Cyclodextrin

The acute histological effects of methylated β-cyclodextrin on the epithelium of the nasal cavity has been investigated in rats using light microscopy.[80] After a single nasal administration of 2% randomly methylated β-cyclodextrin, only minor changes were observed in the appearance of the cilia and the apical cell membranes, and small amounts of mucus were excreted into the nasal cavity. These effects were similar to those noted for control animals dosed with physiological saline (0.9% NaCl). Using confocal laser scanning microscopy, no changes in nasal epithelial cell morphology were observed after a single intranasal administration of 2% randomly methylated β-cyclodextrin, whereas 1 % sodium taurodihydrofusidate resulted in swelling of the cells and substantial mucus extrusion.

Human

Branched - Unmodified

Glucomannan

The inhalation of konjac dust in factories producing konnyaku, a popular food in Japan made from konjac tubers, has been reported to produce allergic bronchial asthma (known as konnyaku asthma) in sensitized individuals.[81] Furtherhmore, bronchial asthma that was likely triggered by the inhalation of Maiko powder has been associated with residents near a konjac milling plant in Japan.[79] Konjac root is dried and ground into powder in the process of manufacturing the food known as konjac. Maiko is a fine konjac root powder that is blown by air pressure to obtain konjac powder for commercial use.

EPIDEMIOLOGY

Linear Polysaccharides and Their Salts

Carrageenan, Agar, and Alginate

An epidemiology study was performed to examine the hypothesis that the increasing incidence of mammary carcinoma in the United States in the twentieth century may be related to the consumption of carrageenan and possibly other water-soluble polymers.[82] A time-trend analysis using age-adjusted incidence data and consumption data from established sources was used to test this hypothesis. Statistical analysis, using Pearson and Spearman correlation coefficients, was performed to identify associations between water-soluble polymer

consumption and cancer incidence. Lag periods of 10, 15, 20, 25, 30, and 35 years were introduced to consider a latent effect between intake and the occurrence of breast cancer.

At least 4 values for consumption and corresponding incidence were required for inclusion in the correlation analysis. Consumption data on the polysaccharide gums studied were reported as pounds/person/year. These water-soluble polymer utilization data, obtained from several libraries throughout the United States, were predominantly from published data compiled as research for the food industry. For carrageenan, 80% of total consumption was identified as food consumption, and the remainder was attributed to products such as toothpaste, deodorants, room deodorizers, etc. Food consumption data on other gums were as follows: sterculia urens gum (< 10%), agar (50%), alginates (60%), and pectin (80 to 95%). Incidence data for breast cancer were obtained from published sources and were presented as the age-adjusted incidence data per 100,000 population using the 1970 census data.

The following positive correlations between gum consumption and the incidence of mammary carcinoma were found. For carrageenan, positive correlations (statistically significant) were found at 25 years ($r = 0.88$; $P = 0.048$) and 30 years ($r = 0.96$; $P = 0.042$). The Spearman correlation coefficient for carrageenan at 30 years was also statistically significant ($r = 1.0$; $P < 0.0001$). Statistically significant positive correlations were also reported for alginate (at 30-year lag period) and agar (at 10- and 25-year lag periods). The Spearman correlation coefficient was significant for pectin at at 30 years. Sterculia urens gum did not demonstrate any statistically significant correlations. This analysis demonstrated that polysaccharide gum consumption correlated positively with increased incidence of breast carcinoma.

Branched - Unmodified

Pectin and Sterculia Urens Gum

Epidemiology data on pectin and sterculia urens gum are included in the preceding study on carrageenan, agar and alginate.[82]

MISCELLANEOUS STUDIES

Endocrine Function and Vitamin D Absorption

Branched - Unmodified

Glucomannan

A double-blind trial on the efficacy of glucomannan in the treatment of pediatric obesity was performed.[83] The study involved 60 children under the age of 15 (mean age: 11.2 years; mean overweight: 46%). Thirty children received 1 g of glucomannan twice daily for two months, and the other 30 children received a placebo according to the same schedule. Clinical side effects were evaluated in both groups by measuring indicators of intestinal absorption, lipid metabolism, and thyroid and adrenocortical function. When the 2 groups were compared, there were no significant differences in intestinal absorption, thyroid or adrenocortical function, or clinical symptoms. However differences in lipid metabolism were significant. The treated group had decreased α-lipoprotein and increased pre-β-lipoprotein and triglyceride. The authors suggested that the metabolic alteration observed may have been due to a primary decrease in α-lipoprotein, most likely because of inadequate water intake. It was noted that these study results question the efficacy of glucomannan in the treatment of childhood obesity.

Antifungal Activity

Linear Polysaccharides and Their Salts

Calcium Alginate

The antifungal properties of calcium alginate fiber were studied using *Candida albicans*.[48] Fungal inhibitory rates were measured using the plate-count method, following the shake-flask test. Additionally, an inhibition-zone test and observation by scanning electron microscopy were performed. The inhibitory rate of calcium alginate fibers was 49.1%, and was classified as weak when compared to zinc alginate (92.2% inhibitory rate). The inhibitory rate was calculated using the following equation: Inhibitory rate = [(A – B)/A] x 100%. A was defined as the number of fungal colony on blank control plates. B was defined as the number of fungal colony on test plates.

Muscle Inflammation

Linear Polysaccharides and Their Salts

Carrageenan

Local muscle inflammation was induced by injecting carrageenan (10 mg/kg) into the right tibialis anterior muscle in 22 healthy ARC mice (6 weeks old).[84] The contralateral muscle was injected with sterile isotonic saline, and the muscles were removed after 24 h for measurement of contractile function and cytokine concentration. Carrageenan significantly reduced maximum specific force, decreased the maximum rate of force development, altered the force-frequency relationship, and increased intramuscular levels of pro-inflammatory cytokines and chemokines. These results indicate that injected carrageenan directly affects contractile function and causes skeletal muscle weakness.

Anti-inflammatory/Antioxidant Activity

Linear Polysaccharides and Their Salts

Alginic Acid

Alginic acid, isolated from brown algae (*Sargassum wightii*), was evaluated in a study involving groups of 6 arthritic adult male Sprague-Dawley rats.[85] The oral dosing of alginic acid (100 mg/kg) in arthritic rats reduced paw edema and the activities of enzymes such as cyclooxygenase, lipoxygenase and myeloperoxidase. Reduction in the level of C-reactive protein, ceruloplasmin, and rheumatoid factor were also observed in arthritic rats treated with alginic acid. Additionally, reduced lipid peroxidation and enhanced activities of antioxidant enzymes were reported, which suggest the antioxidant potential of the compound. Histopathological analysis indicated that alginic acid treatment reduced paw edema and inflammatory infiltration in arthritic rats. Overall, study results suggest that alginic acid isolated from *Sargassum wightii* exhibits potent anti-inflammatory and antioxidant activity.

SUMMARY

The polysaccharide gums are naturally derived materials that comprise polysaccharides obtained from plants or algae. As a group, they comprise polymers of simple saccharide monomers. Based on the different chemical structures that are associated with polysaccharide gums, these ingredients can be subdivided into categories such as modified, unmodified, linear, branched, and cyclic. Many of the polysaccharide gums reviewed in this safety assessment function as viscosity increasing agents in cosmetic products. According to information supplied to the FDA by industry as part of the VCRP and results from a Council survey of ingredient use concentrations, 59 polysaccharide gums are being used in cosmetic products.

The Council survey data also indicate that polysaccharide gums are being used in cosmetics at maximum ingredient use concentrations up to 50% (i.e., for algin in paste masks and mud packs). Polysaccharide gums are used at concentrations up to 9.5% (avena sativa (oat) starch) in cosmetic products that are sprayed, which also includes use in a pump hair spray at a maximum concentration of 0.45% (corn starch modified), and at concentrations up to 45.7% (corn starch modified) in cosmetic products that possibly are sprayed. Additionally,

polysaccharide gums are used in cosmetic products (powders) at concentrations up to 33% (tapioca starch). Because polysaccharide gums are used in products that are sprayed, they could possibly be inhaled.

Maltodextrin, the most frequently used cosmetic ingredient reviewed in this safety assessment, is prepared as a white powder or concentrated solution by partial hydrolysis of corn starch, potato starch, or rice starch. It is an approved direct food additive affirmed as GRAS by the FDA. The following other polysaccharide gums reviewed in this safety assessment have also been classified as GRAS direct food additives: agar, alginic acid, ammonium alginate, amylose (i.e., high amylose corn starch is GRAS), calcium alginate, pectin, potassium alginate, dextrin, solanum tuberosum (potato) starch, starch acetate, tapioca starch, hydroxypropyl starch, propylene glycol alginate, ghatti gum, and sterculia urens gum.

In 2014, the JECFA concluded that the use of carrageenan in infant formula or formula for special medical purposes at concentrations up to 1,000 mg/L is not of concern.

Data on native carrageenans extracted from different types of algae indicate that different types of carrageenan have reasonable stability to heating at 75°C down to pH 4, and that the rate of depolymerization increases dramatically as the pH decreases from 4 to 3. These data indicate the susceptibility of carrageenan to acid hydrolysis under certain conditions.

The results of a percutaneous absorption study involving hairless mouse skin indicate that 2-hydroxypropyl-β-cyclodextrin had extremely low permeability, approximately 0.02% of the amount applied to the skin.

In studies involving rats, there was no specific accumulation of orally administered cyclodextrin in organs, and it was rapidly hydrolyzed to maltose and glucose. In another study, 95% of ingested sterculia urens gum was excreted in the feces of rats. Carrageenan was not degraded or absorbed from the gastrointestinal tract of rodents, dogs, and non-human primates, and rapid and nearly complete enzymatic degradation of starch acetate was reported. Dietary sterculia urens gum was neither digested nor degraded by enteric bacteria in humans, which is similar to what was observed in rats. In a human oral feeding study on tapioca starch, a rapid increase in plasma glucose was observed after dosing.

An LC_{50} > 0.0015 mg/l was reported for glucomannan in an acute inhalation toxicity study involving rats. The transbronchial injection of 0.75% carrageenan (in physiological saline) induced pneumonia in rabbits.

Acute oral dosing of rats with sterculia urens gum at a dose of 10 g/kg body weight did not cause death, and the same was true for rats dosed with 5,000 mg/kg potato starch modified, 5,000 mg/kg calcium starch isododecenylsuccinate (considered structurally similar to sodium hydrolyzed potato starch dodecenylsuccinate and corn-starch modified), 2,000 mg/kg corn starch modified, 2,000 mg/kg dextrin palmitate, 2,000 mg/kg dextrin myristate, or 2,000 mg/kg stearoyl inulin. Acute oral LD_{50} values of > 2,800 mg/kg body weight (mice) and > 5,000 mg/kg body weight (rats) have been reported for glucomannan.

In acute dermal toxicity studies on corn starch modified, potato starch modified, dextrin myristate, and dextrin palmitate, an LD_{50} of > 2,000 mg/kg (rats) was reported. The same results were reported for glucomannan in an acute dermal toxicity study involving rabbits.

Repeated dose oral toxicity studies on the following were performed: algin (25% in diet, mice) starch acetate (55% in diet, mice), arabinoxylan (~ 80% arabinoxylan oligopeptides in wheat bran extract [extract test concentrations up to 7.5% in diet], rats), inulin (7.5% in diet, rats), carboxymethyl inulin (31.1% aqueous at doses up to 1,000 mg/kg/day, rats), carrageenan (up to 5% in diet [rats]; up to 25% in diet [mice]; up to 500 mg/kg/day [monkeys]), cyclodextrin (up to 50,000 ppm in diet [rats]; up to 20% in diet [dogs]), ghatti gum (up to 5% in diet, rats), glucomannan (up to 8% in diet, rats), pectin (up to 10% pectin-derived acid oligosaccharides in diet, rats), solanum tuberosum (potato) starch (up to 10% in diet, rats), and sterculia urens gum (5 g/kg/day, rats; 7% in diet, rats). Sodium alginate was nephrotoxic in mice, but results for starch acetate were of little, if any, toxicological significance. The NOAEL for wheat bran extract in rats was 4.4 g/kg/day, the highest dose administered; there were no remarkable findings in control rats dosed with inulin. There were no toxicologically significant findings in rats dosed with carboxymethyl inulin, and the same was true for ghatti gum. The liver and kidney were identified as

target organs for toxicity in rats dosed with β-cyclodextrin, but there was no evidence of systemic toxicity in dogs. There were no treatment-related effects in dogs dosed with γ-cyclodextrin. Treatment-related histopathological changes in the urinary bladder were observed in rats fed pectin-derived acidic oligosaccharides in the diet. No adverse effects were observed in rats dosed repeatedly with sterculia urens gum. Transient fatty degeneration, with focal necrosis of the liver was observed in rats fed glucomannan in the diet.

Repeated oral feeding of humans with propylene glycol alginate (up to 200 mg/kg/day) or sterculia urens gum (10.5 g in diet/day) did not cause toxicity.

Systemic toxicity was not observed in guinea pigs that received repeated dermal applications of 31.1% aqueous carboxymethyl inulin, or in rats dosed dermally (2 g/kg body weight/day) with potato starch modified.

There were no changes in cell morphology of the nasal epithelium of rats after intranasal administration of methyl cyclodextrin.

Pathological lesions of the ocular mucosa were not observed after 2% algin was instilled into the eyes of rabbits. Carrageenan was non-irritating to the unrinsed eyes of rabbits, but was minimally irritating to rinsed eyes. Ocular irritation was not observed in rabbits tested with dextrin myristate, dextrin palmitate, or stearoyl inulin. An eye gel containing 2.45% maltodextrin was classified as a non-irritant in the *in vitro* bovine corneal opacity and permeability assay, and in the *in vitro* EPI-Ocular® assay. Corn starch modified and calcium starch isododecenylsuccinate (considered structurally similar to sodium hydrolyzed potato starch dodecenylsuccinate and corn-starch modified) were minimally irritating to the eyes of rabbits. Potato starch modified and a 16.8% aqueous suspension of potato starch modified were slightly irritating to the eyes of rabbits. Hydroxypropyltrimonium hydrolyzed corn starch had practically no irritation potential at concentrations of 5%, 10%, and 15% in the *in vitro* HET-CAM ocular irritation assay. Mixtures containing 1.35% hydrolyzed furcellaran were classified as slightly toxic in a cytotoxicity assay involving cultured fibroblasts, and this finding was classified as almost non-irritating to slightly irritating to the eyes.

In a primary skin irritation study, results were negative for 2% algin in rabbits. In a cumulative skin irritation study involving rabbits, the results observed at macroscopic or microscopic examination indicated that 2% algin did not induce a severe reaction. Potato starch modified (10% solids aqueous solution) caused minimal to slight acanthosis in rabbits, and a 50% slurry of calcium starch isododecenylsuccinate (considered structurally similar to sodium hydrolyzed potato starch dodecenylsuccinate and corn-starch modified) was mildly irritating to the skin of rabbits. At a dose of 2,000 mg/kg in an acute dermal toxicity study, corn starch modified (30% solids in distilled water) was classified as a mild skin irritant in rabbits.

Skin irritation was not observed in albino guinea pigs patch-tested with 100% carboxymethyl inulin. Erythema and edema were observed in an acute dermal toxicity study involving rats dosed with 2 g/kg potato starch modified; all reactions cleared by 72 h. Neither erythema nor edema was observed in rats that received repeated dermal applications of the same dose of potato starch modified. Dextrin palmitate or dextrin myristate did not cause skin irritation in rabbits or skin sensitization in guinea pigs evaluated in the maximization test. A trade name mixture containing 1.35% hydrolyzed furcellaran was classified as non-irritating to the skin of human subjects. A trade name mixture containing 0.6% hydrolyzed furcellaran was classified as non-irritating and non-sensitizing when applied to the skin of human subjects.

In the guinea pig maximization test, corn starch modified (20% solution) and 31.1% aqueous carboxymethyl inulin did not induce sensitization. In the Buehler test for skin sensitization, potato starch modified (18.5% aqueous suspension) caused faint erythema during induction, but there was no evidence of sensitization in animals tested. Also, in the Buehler test, a paste of 50% calcium starch isododecenylsuccinate (considered structurally similar to sodium hydrolyzed potato starch dodecenylsuccinate and corn-starch modified) was not a sensitizer in guinea pigs. ι-Carrageenan and konjac flour (glucomannan is primary polysaccharide component; the antigen is an acidic protein [AG40D-2]) were also non-sensitizing to the skin of guinea pigs.

Corn starch modified (7.5%) did not induce cumulative skin irritation in 26 subjects or skin sensitization in 113 subjects tested. A 50% w/v slurry or 50% solids slurry of calcium starch isododecenylsuccinate (considered

structurally similar to sodium hydrolyzed potato starch dodecenylsuccinate and corn-starch modified) was classified as a probable mild irritant in a 21 day cumulative skin irritation study involving 23 human subjects.

Algae exopolysaccharides (1%) did not cause skin irritation or sensitization in an HRIPT involving 50 subjects. An eye gel containing 2.45% maltodextrin did not induce allergic contact dermatitis in an HRIPT involving 103 subjects. Results were negative for skin irritation and allergic contact dermatitis in 12 male subjects patch-tested with 20% aqueous sodium alginate. Negative results for skin sensitization were also reported for 227 subjects in a human RIPT on a cleanser containing 10 wt% sodium hydrolyzed potato starch dodecenylsuccinate. Neither skin irritation nor sensitization was observed in the following HRIPT's: 54 subjects tested with a rinse-off facial product containing 42.69% dextrin, 51 subjects tested with a leave-on facial product containing 0.3% dextrin myristate, and 47 subjects tested with hydroxypropyltrimonium hydrolyzed corn starch (15%).

Allergenicity was not associated with the oral dosing of human subjects with propylene glycol alginate, and dermal application of a calcium alginate dressing to patients did not cause any side effects that were classified as severe.

Sodium hydrolyzed potato starch dodecenylsuccinate was evaluated for phototoxicity at concentrations ranging from 68.1 to 1,000 µg/ml in the *in vitro* neutral red uptake phototoxicity assay (BALB/3T3 clone A31 mouse embryo fibroblast cultures). The test material was not considered to have phototoxicity potential.

The concentration of konjac flour that caused a 50% decrease in respiratory rate (RD_{50}) in mice in a sensory irritation evaluation was 110 mg/m^3. In a subsequent study, the criteria used to define respiratory tract sensitization (increase in respiratory rate of 36% and change in respiratory waveform) were achieved in 25% of the 8 guinea pigs challenged with konjac flour (mean exposure concentration range = 50 to 68 mg/m^3). The inhalation of konjac dust in factories producing konnyaku, a popular food in Japan made from konjac tubers, has been reported to produce allergic bronchial asthma in sensitized individuals.

In studies evaluating effects on the immune system, an acidic polysaccharide produced by *Polianthes tuberosa* cells was classified as an immunosuppressive polysaccharide. The injection (i.p.) of potassium carrageenan into rats resulted in significant elevation of serum IgM, but not IgG.

In pregnant mice that received doses of kappa/lambda-carrageenan (from *C. crispus*, sodium or calcium salt) at oral doses up to 900 mg/kg/day during gestation, there was a dose-dependent decrease in the number of live pups and in pup weight. Skeletal maturation was also retarded. In another study in which pregnant mice received oral doses of the same test substance (sodium or calcium salt) at doses up to 600 mg/kg/day during gestation, there was a dose-dependent increase in the incidence of missing skeletal sternebrae. However, feeding with the test substance (calcium salt) at dietary concentrations up to 5% prior to mating in a three-generation feeding study, no specific external, skeletal, or soft-tissue anomaly could be correlated with dosage. In a study in which calcium carrageenan was fed at dietary concentrations up to 1.8% prior to mating, during breeding, and throughout gestation, lactation, and post-weaning, there were no differences between test and negative control groups regarding length of gestation, litter size, or sex distribution.

The oral dosing of pregnant hamsters with doses of kappa/lambda-carrageenan (from *C. crispus*, sodium or calcium salt) up to 600 mg/kg/day during gestation resulted in some evidence of a dose-dependent delay in skeletal maturation. In a similar study in which hamsters received oral doses of the test substance (sodium or calcium salt) up to 200 mg/kg/day during gestation, there were no dose-related teratogenic or fetotoxic effects. When pregnant rabbits were dosed orally with the test substance (sodium or calcium salt) at doses up to 600 mg/kg/day during gestation, the numbers of skeletal or soft tissue abmormalities did not differ from those of controls.

Neither reproductive nor developmental toxicity was observed in rat dietary feeding studies on cyclodextrin (up to 20%), and pectin-derived acidic oligosaccharides (10%). Sterculia urens gum was not teratogenic when administered in a corn oil suspension to rats (doses up to 900 mg/kg/day) rabbits (doses up to 635 mg/kg/day) or mice (doses up to 170 mg/kg/day) during gestation. Cyclodextrin also did not cause reproductive or developmental toxicity in rabbits when administered at dietary concentrations up to 20%, and the same was true when pregnant cats were fed 2% glucomannan in the diet during gestation.

In bacterial assays, the following were not genotoxic either with or without metabolic activation: arabinoxylan, carboxymethyl inulin, carrageenan, corn starch modified, ghatti gum, glucomannan, a trade name mixture containing 0.6% hydrolyzed furcellaran, pectin-derived acidic oligosaccharides, calcium starch isododecenylsuccinate (considered structurally similar to sodium hydrolyzed potato starch dodecenylsuccinate and corn-starch modified), and a sodium hydrolyzed potato starch dodecenylsuccinate tradename material. In mammalian assays with and without metabolic activation, wheat bran extract, carboxymethyl inulin, carrageenan, ghatti gum, and glucomannan were not genotoxic. However, results for pectin-derived acidic oligosaccharides in mammalian assays were either equivocal or it was classified as clastogenic. Sterculia urens gum was not genotoxic in cytogenetic assays (*in vitro* and *in vivo*) or in the *in vivo* dominant lethal gene test.

Agar, isolated from Pterocladia, was not carcinogenic in F344 rats or $B6C3F_1$ mice that received concentrations of 25,000 ppm or 50,000 ppm in the diet. Neither algin (25% in diet) nor starch acetate (55% in diet) was found to be carcinogenic in an oral feeding study involving mice. When fed in the diet to rats, carrageenan (up to 25% in diet), and cyclodextrin (up to 675 mg/kg/day), also were not carcinogenic. Carrageenan (up to 5% in diet) was not carcinogenic when fed to hamsters. In a co-carcinogenicity study, carrageenan (15% in the diet) enhanced the incidence of colon tumors in female Fischer 344 rats injected with azoxymethane or *N*-nitrosomethylurea.

Colorectal tumors were found in Sprague-Dawley rats fed 5% or 10% degraded carrageenan, but not 1% degraded carrageenan, in the diet for up to 24 months. Colorectal tumors were also observed in Sprague-Dawley rats that received 5% degraded carrageenan in drinking water for 15 months, and in Sprague-Daweley rats dosed with 1 g/kg or 5 g/kg degraded carrageenan by gastric intubation for 15 months. Fischer 344 rats that received 10% degraded carrageenan in the diet for up to 9 months also had colorectal tumors.

The feeding of rats with an inulin-enriched diet (10% in diet) resulted in the promotion of adenoma growth. Mucosal hyperplasia in the small intestine was observed in rats fed 2.5% pectin in the diet. In another feeding study, 5% methoxylated pectin in the diet increased the multiplicity of colon tumors in rats injected with DMH. In another co-carcinogenicity study, carrageenan (15% in the diet) enhanced the incidence of colon tumors in female Fischer 344 rats injected with azoxymethane or *N*-nitrosomethylurea.

Anticarcinogenic effects have been associated with arabinoxylan and inulin in studies involving rats, with glucomannan in mice, and with konjac flour in rats. The antitumor/anticarcinogenic activity of wheat bran arabinoxylan in mice and arabinoxylan-oligosaccharides in rats has also been reported.

In an epidemiology study, a positive correlation between polysaccharide gum consumption and the incidence of mammary carcinoma was found for carrageenan, alginate, agar, and pectin, but not for sterculia urens gum.

DISCUSSION

The polysaccharide gums comprise polysaccharides obtained from plants or algae. Based on the different chemical structures of polysaccharide gums, these ingredients can be subdivided into categories such as modified, unmodified, linear, branched, and cyclic. Regardless of how they are categorized, the molecular structures of these ingredients are polymers composed of monosaccharides. Based on chemical similarities, relevant data have been included on analogous polysaccharide ingredients. Therein, inference may be appropriate from one ingredient to the next and from one ingredient to one subgroup of polysaccahrides, of which that ingredient or analog is a member.

The substantial molecular sizes of many of these polysaccharides suggest that skin penetration would be unlikely. Specifically, the percutaneous absorption of ^{14}C-2-hydroxypropyl-β-cyclodextrin through intact hairless mouse skin was extremely low, i.e., approximately 0.02% of the amount applied to the skin. Thus, during cosmetic use, these ingredients are unlikely to have significant systemic bioavailability.

The use concentration data provided indicate that algin is being used in cosmetics at concentrations up to 50% (in mud packs). The Expert Panel acknowledged the absence of skin irritation and sensitization data on algin at this concentration, but noted that results were negative when carboxymethyl inulin was tested at concentrations up to 100% in a skin irritation study involving guinea pigs, and the absence of clinically relevant reactions to

polysaccharide gums in dermatologic practice. The Panel is aware of severe granulomatous reactions in patients injected intradermally with an aesthetic injectable filler consisting of purified alginate; however, it was determined that these findings are not relevant to the use of alginates as cosmetic ingredients. Furthermore, systemic toxicity is not a concern in relation to repeated exposure to polysaccharide gums during cosmetic use, considering the absence of gross or microscopic changes in monkeys dosed orally/fed carrageenan in the diet for 7.5 years.

Genotoxicity data for pectin-derived acidic oligosaccharides in mammalian assays were equivocal, but some were classified as clastogenic. However, the Panel noted that clastogenicity was observed only at highly cytotoxic concentrations. The Panel reviewed data indicating that degraded carrageenan (also known as poligeenan) in the diet induced colorectal tumors in rats. Degraded carrageenan used in those studies was produced by acid hydrolysis of a certain type of seaweed. In light of this information and the colon carcinogenicity data, the Panel expressed concern about the use of hydrolyzed carrageenan as a cosmetic ingredient, in the absence of data demonstrating that hydrolyzed carrageenan is chemically dissimilar to poligeenan and does not share its carcinogenic properties. Thus, the Panel determined that method of manufacture and impurities data are needed to determine the safety of hydrolyzed carrageenan in cosmetic products.

Polysaccharide gums are used at concentrations up to 9.5% (avena sativa (oat) starch) in perfumes, at a maximum concentration of 0.45% (corn starch modified) in pump hair sprays, and at concentrations up to 33% (tapioca starch) in powders. The available data indicate that food grade konjac flour (primary polysaccharide component is glucomannan) induced sensory irritation of the respiratory tract in mice and respiratory tract sensitization in guinea pigs. Furthermore, the inhalation of konjac dust in factories in Japan has produced allergic bronchial asthma in sensitized individuals. Additional research suggested that the purified antigen AG40D-2 (acidic protein) was responsible for the respiratory sensitization observed, and that this effect was not attributed to glucomannan. Transbronchial injection of 0.75% carrageenan (in physiological saline) induced pneumonia, followed by emphysema, in rabbits. In consideration of these data, the Panel discussed the potential for incidental inhalation exposures to polysaccharide gums in products that are sprayed or in powder form and agreed that, based on likely airborne particle size distributions and concentrations in the breathing zone and ingredient use, incidental inhalation would not lead to local respiratory effects or systemic effects.

The Panel expressed concern about pesticide residues and heavy metals that may be present in ingredients txhat are derived from plants. They stressed that the cosmetics industry should continue to use current good manufacturing practices (cGMPs) to limit impurities. The Panel also agreed that the same suggestion is applicable to alkylating and other agents (e.g., haloethylaminopropionic acid; 3-(dodecenyl)-2,5-furandione; and 2,3-epoxypropyltrimethylammonium chloride) that are used to modify polysaccharide gums.

CONCLUSION

The CIR Expert Panel concluded that the following 105 ingredients are safe in the present practices of use and concentration in cosmetics, as described in this safety assessment, and that the available data are insufficient for determining the safety of hydrolyzed carrageenan in cosmetic products.

Linear Polysaccharides and Their Salts

Agar
Agarose
Algin
Alginic Acid
Ammonium Alginate*
Amylose*
Astragalus Gummifer Gum
Calcium Alginate
Calcium Carrageenan*
Carrageenan
Magnesium Alginate*
Mannan
Polianthes Tuberosa Polysaccharide
Potassium Alginate
Potassium Carrageenan*
Sodium Carrageenan
TEA-Alginate*

Linear -Modified

Amylodextrin
Hydrolyzed Furcellaran*
Maltodextrin
Sodium Algin Sulfate*

Branched -Unmodified

Amylopectin*
Aphanothece Sacrum Polysaccharide*
Arabinoxylan*
Avena Sativa (Oat) Starch
Cichorium Intybus (Chicory) Root Oligosaccharides
Galactoarabinan
Ghatti Gum*
Glucomannan
Inulin
Pectin
Phaseolus Angularis Seed Starch*
Phaseolus Radiatus Seed Starch*
Pisum Sativum (Pea) Starch*
Pueraria Lobata Starch
Solanum Tuberosum (Potato) Starch
Starch Acetate
Sterculia Urens Gum
Tamarindus Indica Seed Gum
Tapioca Starch
Triticum Vulgare(Wheat) Starch
Xyloglucan*

Branched - Modified

Calcium Starch Isododecenylsuccinate*
Calcium Starch Octenylsuccinate*
Corn Starch Modified
Dextrin
Dextrin Behenate*
Dextrin Isostearate*
Dextrin Laurate*
Dextrin Myristate
Dextrin Palmitate
Dextrin Palmitate/Ethylhexanoate
Dextrin Stearate*
Glyceryl Alginate
Glyceryl Dimaltodextrin*
Glyceryl Starch
Hydrolyzed Pectin
Hydroxypropyltrimonium Hydrolyzed Corn Starch
Hydroxypropyltrimonium Hydrolyzed Wheat Starch
Hydroxypropyl Oxidized Starch*
Hydroxypropyl Starch
Hydroxypropyltrimonium Maltodextrin Crosspolymer
Laurdimonium Hydroxypropyl Hydrolyzed Wheat Starch
Palmitoyl Inulin*
Potassium Dextrin Octenylsuccinate*
Potassium Undecylenoyl Alginate*
Potassium Undecylenoyl Carrageenan*
Potato Starch Modified
Propylene Glycol Alginate
Sodium Carboxymethyl Inulin*
Sodium Carboxymethyl Starch
Sodium Dextrin
Octenylsuccinate*
Sodium Hydrolyzed Potato Starch Dodecenylsuccinate
Sodium Hydroxypropyl Oxidized Starch Succinate*
Sodium Oxidized Starch Acetate/Succinate
Sodium Starch Octenylsuccinate
Sodium/TEA-Undecylenoyl Carrageenan*
Sodium/TEA-Undecylenoyl Alginate*
Starch Acetate/Adipate*
Starch Diethylaminoethyl Ether
Starch Hydroxypropyltrimonium Chloride
Starch Laurate*
Starch Tallowate*
Stearoyl Inulin
Tapioca Starch Crosspolymer*
TEA-Dextrin Octenylsuccinate*
Undecylenoyl Inulin*

Cyclic

Cyclodextrin
Cyclotetraglucose*

Cyclic - Modified

Hydroxyethyl Cyclodextrin
Hydroxypropyl Cyclodextrin
Cyclodextrin Hydroxypropyltrimonium Chloride*
Cyclodextrin Laurate
Methyl Cyclodextrin

Unknown Structural Configuration

Algae Exopolysaccharides*
Cassia Angustifolia Seed Polysaccharide*
Prunus Persica (Peach) Gum*

Unknown Structural Configuration - Modified

Hydrogenated Potato Starch*
Hydrogenated Starch Hydrolysate
Hydrolyzed Corn Starch Hydroxyethyl Ether*
Hydrolyzed Corn Starch Octenylsuccinate
Hydrolyzed Soy Starch*
Hydrolyzed Starch
Hydrolyzed Triticum Spelta Starch*
Hydrolyzed Wheat Starch

*Not reported to be in current use. Were ingredients in this group not in current use to be used in the future, the expectation is that they would be used in product categories and at concentrations comparable to others in this group.

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
[*Italicized text* and all structures below have been added by CIR staff.]

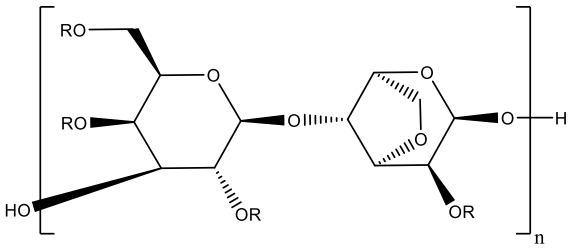
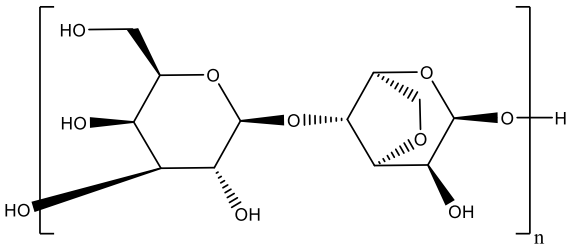
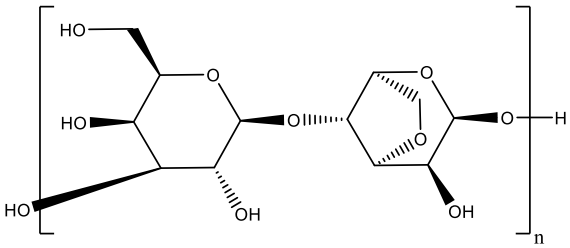
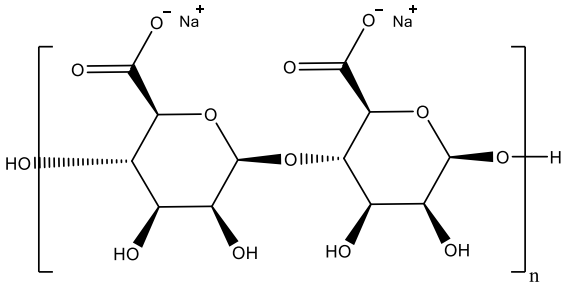
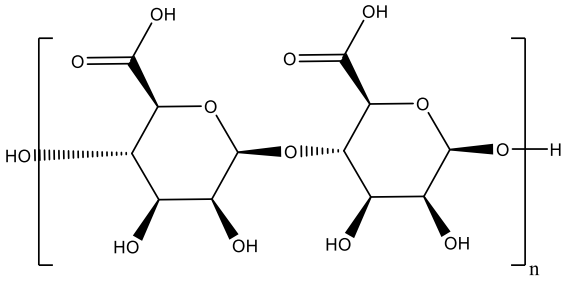
Ingredient CAS No.	Definition	Formula/structure
Linear polysaccahrides and their salts		
Agar 9002-18-0	Agar is the dried, hydrophilic, colloidal polygalactoside derived from various Gelidium species or closely related red alga. *Agar is typically a mixture of agarose and agaropectin.*[86]	 and  wherein R is hydrogen, sulfate, or pyruvate
Agarose 9012-36-6	Agarose is the polysaccharide extracted from the red seaweed Gracilaria.	
Algin 9005-38-3	Algin is the sodium salt of Alginic Acid. *Alginic Acid is the carbohydrate obtained by the alkaline extraction of various species of brown seaweed, Phaeophyceae.* Other source: Algin is a linear polymer of anhydro-β-D-mannuronic acid. The main structural feature of this molecule is a chain of 1,4-linked-β-D-mannuronic acid residues.[87]	
Alginic Acid 9005-32-7	Alginic Acid is the carbohydrate obtained by the alkaline extraction of various species of brown seaweed, Phaeophyceae. *Alginic acid is a polysaccharide comprised of 1,4-linked-β -D-mannuronic and α-L-guluronic acids.*[88]	

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
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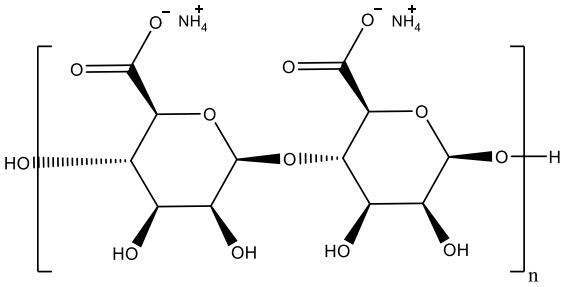
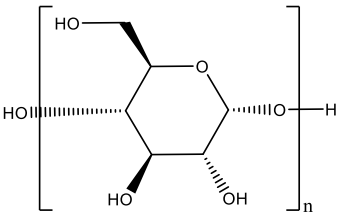
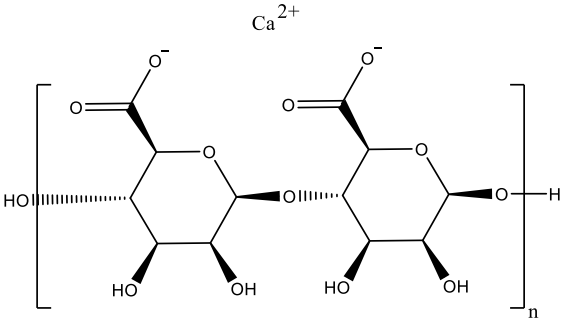
Ingredient CAS No.	Definition	Formula/structure
Ammonium Alginate 9005-34-9	Ammonium Alginate is the ammonium salt of Alginic Acid. *Alginic Acid is the carbohydrate obtained by the alkaline extraction of various species of brown seaweed, Phaeophyceae.* Other sources: Alginate, a term that refers to salts and derivatives of alginic acid, is a gelling polysaccharide and a structural component extracted from marine brown algae (*Phaeophyceae*), in which it is present in the cell wall as water-insoluble salts.[89] Alginates are polymers composed of β-1,4-D-mannuronic acid (M) and α-1,4-L-guluronic acid (G). Alginates have been determined to be true block copolymers, organized in homopolymeric blocks consisting of either mannuronate or guluronate, or mixed in heteropolymeric MG-block structures. Alginate, the monovalent salt form of alginic acid, is a non-repeating copolymer that contains two uronic acid monomers, 1,4- linked-β-D-mannuronic and α-L-guluronic acid.[90] These residues exist in linear polysaccharide chains that can dimerize to form hydrogels at room temperature in the presence of divalent ions such as calcium.	
Amylose 9005-82-7	Amylose is the carbohydrate stored by plants that consists of a linear (1→4)-(structure)-D-glucan polymer. Other source: Starch is composed of two polysaccharides, amylose and amylopectin.[91] Amylose is a complex α-glucan. It is an essentially linear polymer made up of α(1-4)-linked glucopyranose units.	
Astragalus Gummifer Gum	Astragalus Gummifer Gum is a dried resinous exudate obtained from Astragalus gummifer. It *is a complex polysaccharide composed of D-galacturonic acid, D-galactose, D-xylose, and L-arabinose, with associated calcium, and potassium cations.*	
Calcium Alginate 9005-35-0	Calcium Alginate is the calcium salt of Alginic Acid. *Alginic Acid is the carbohydrate obtained by the alkaline extraction of various species of brown seaweed, Phaeophyceae.*	
Calcium Carrageenan 9049-05-2	Calcium Carrageenan is the calcium salt of Carrageenan.	
Carrageenan 9000-07-1	Carrageenan is the plant material obtained from various members of the *Gigartinaceae* or *Solieriaceae* families of the red seaweed, *Rhodophyceae*. Other sources: Carrageenan is a high-molecular-weight sulfated polygalactan derived from several species of red seaweeds of the class *Rhodophyceae*.[35] Native carrageenan is defined as a hydrocolloid isolated from red algae (seaweed) and consisting mainly of varying amounts (depending on the processing methods) of the ammonium, calcium, magnesium, potassium or sodium salts of sulfate esters of galactose and 3,6-anhydrogalactose copolymers (the two hexose units are alternately linked α-1,3 and β-1,4 in the polymer).[92] A product called 'degraded carrageenan' has been produced from extracts of *Eucheuma spinosum* seaweed by treatment with dilute hydrochloric acid. The most common forms of carrageenan are designated as kappa-, iota-, and lambda carrageenans.[93] Kappa carrageenan is mostly the alternating polymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose. Iota carrageenan is similar, but with the 3,6-anhydro-D-galactose sulfated at the 2-hydroxyl. Between kappa and iota carrageenan, there is a continuum of intermediate compositions that differ only in the degree of sulfation at the 2-OH. Lambda carrageenan has alternating monomeric units composed mostly of D-galactose-2-sulfate (1,3- linked) and D-galactose-2,6-disulfate (1,4-linked).	

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
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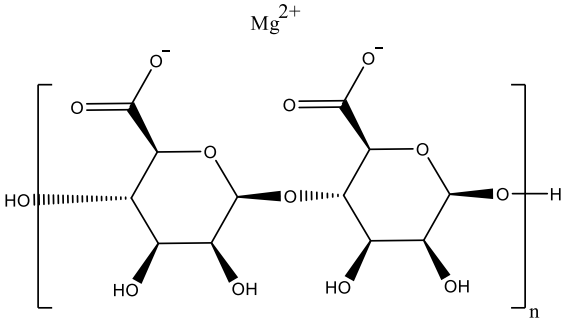
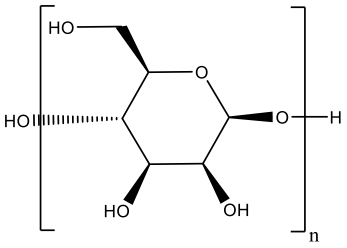
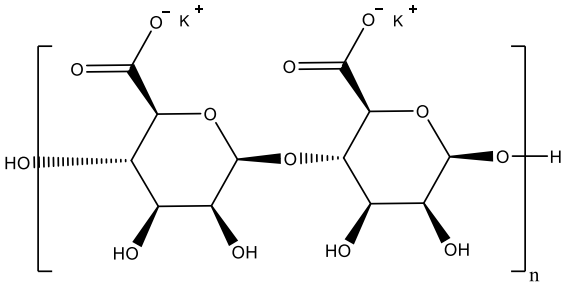
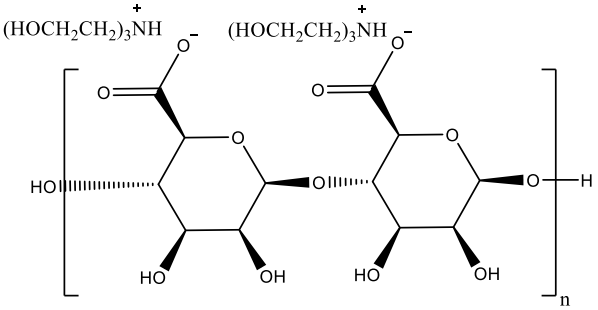
Ingredient CAS No.	Definition	Formula/structure
Magnesium Alginate 37251-44-8	Magnesium Alginate is the magnesium salt of Alginic Acid.	
Mannan 9036-88-8 51395-96-1	Mannan is a natural polysaccharide consisting of a polymer of Mannose.	
Polianthes Tuberosa Polysaccharide	Polianthes Tuberosa Polysaccharide is the polysaccharide fraction produced by the cultured cells of *Polianthes tuberosa.*	
Potassium Alginate 9005-36-1	Potassium Alginate is the potassium salt of Alginic Acid.	
Potassium Carrageenan 64366-24-1	Potassium Carrageenan is the potassium salt of Carrageenan.	
Sodium Carrageenan 60616-95-7 9061-82-9	Sodium Carrageenan is the sodium salt of Carrageenan.	
TEA-Alginate	TEA-Alginate is the triethanolamine salt of Alginic Acid.	

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
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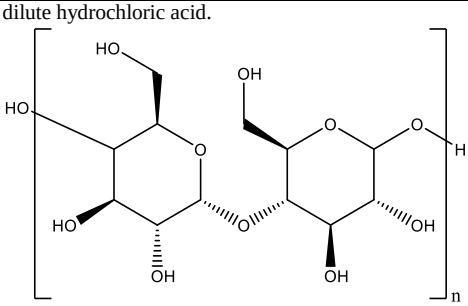
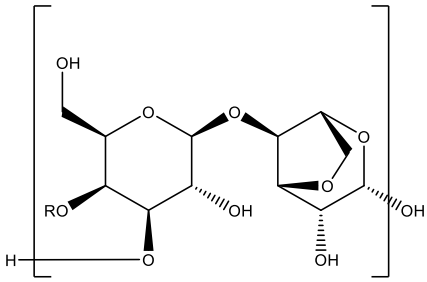
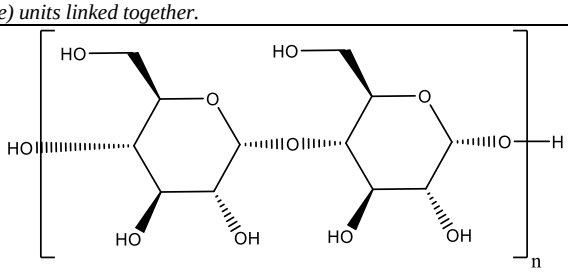
Ingredient CAS No.	Definition	Formula/structure
Linear - modified		
Amylodextrin 9005-84-9	Amylodextrin is the product obtained by treating potato or corn starch with dilute hydrochloric acid.	
Hydrolyzed Carrageenan 53973-98-1	Hydrolyzed Carrageenan is the hydrolysate of Carrageenan derived by acid, enzyme or other method of hydrolysis.	
Hydrolyzed Furcellaran 73297-69-5	Hydrolyzed Furcellaran is the hydrolysate of furcellaran derived by acid, enzyme or other method of hydrolysis. *Furcellaran is composed of D-galactose, 3,6-anhydro-D-galactose and D-galactose- 4-sulfate.* Other source: Information relating to the algal source of hydrolyzed furcellaran indicates that this ingredient is a carrageenan (Kappa type) that is obtained from red algae, *Furcellaria lumbricallis*.[73]	 where R is hydrogen or SO_3^{2-}
Maltodextrin 9050-36-6	Maltodextrin is the saccharide material obtained by hydrolysis of starch. *Maltodextrin is a linear-chain oligosaccharide of glucose, usually obtained from starch by partial, enzymatic treatment*.[94] *The term "maltodextrin" can be applied to any starch hydrolysis product that contains fewer than 20 dextrose (glucose) units linked together.*	
Sodium Algin Sulfate 9010-06-4	Sodium Algin Sulfate is the sulfate ester of Algin.	

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
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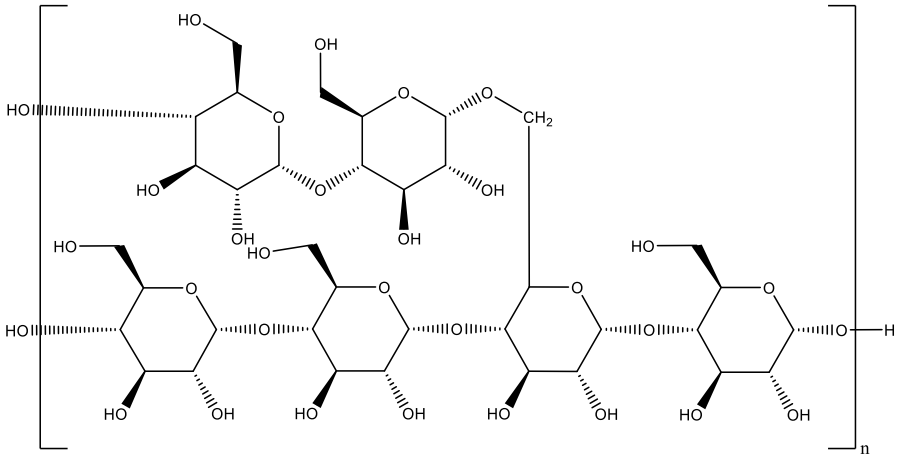
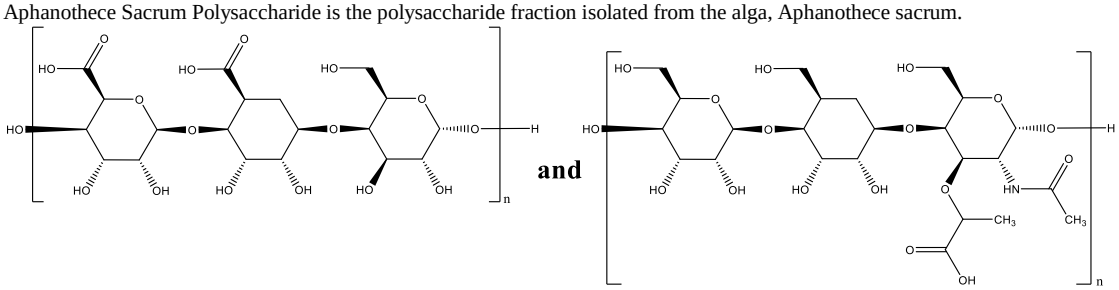
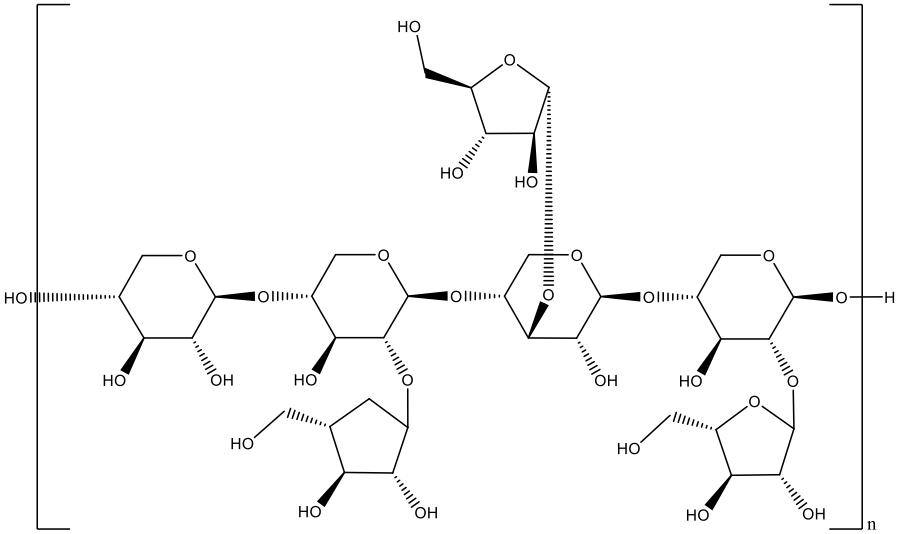
Ingredient CAS No.	Definition	Formula/structure
Branched-unmodified		
Amylopectin 9037-22-3	Amylopectin is the branched chain polysaccharide portion of starch. Other sources: Amylopectin is a complex α-glucan.[91] It is a highly branched polysaccharide composed of segments of linear α(1→4)-linked glucopyranose units joined at branching points via α(1→6) glycosidic linkages to give a structure that resembles a dendrimer. Amylopectin consists of numerous short chains of α(1→4)-linked D-glucopyranosyl residues with a chain length of approximately 6 to 35 units.[95] The chains are α(1→6)-linked into clusters defined as groups of chains, in which the internal chain length between the branches is less than 9 residues.	
Aphanothece Sacrum Polysaccharide	Aphanothece Sacrum Polysaccharide is the polysaccharide fraction isolated from the alga, Aphanothece sacrum.	
Arabinoxylan 9040-27-1	Arabinoxylan is a polysaccharide composed of a xylose backbone with arabinose side chains. Other sources: Arabinoxylan is a non-starch polysaccharide, and is also described as a pentosan.[96] It can also be sub-categorized as water-extractable arabinoxylan and water-unextractable arabinoxylan. Arabinoxylans consist of D-xylopyranosyl residues, connected together by β-(1/4) glycosidic bonds.[97,98] Moreover, acetic acid, hydroxycinnamic acids, ferulic acid, and p-coumaric acid are linked with xylose residues in arabinoxylan.[99,100] The attached moieties are partly or wholly lost when arabinoxylan is extracted from cereal or cereal subfractions using alkaline extraction.[96,101,102]	

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
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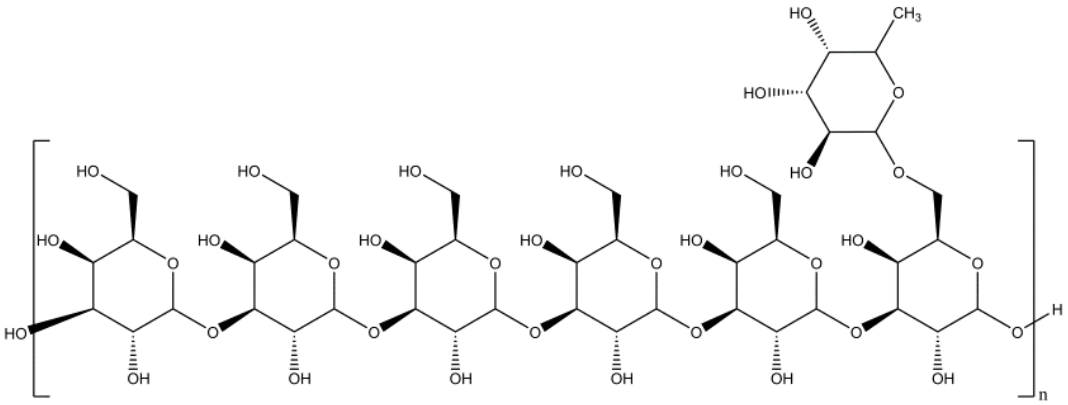
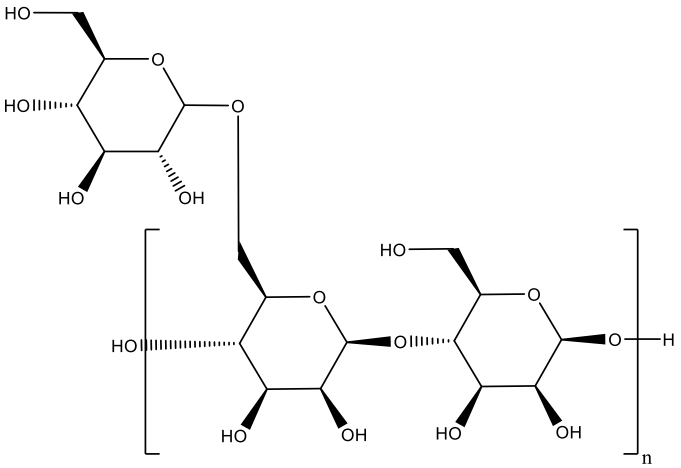
Ingredient CAS No.	Definition	Formula/structure
Avena Sativa (Oat) Starch 9005-25-8 (generic)	Avena Sativa (Oat) Starch is a starch obtained from oats, Avena sativa.	
Cichorium Intybus (Chicory) Root Oligosaccharides	Cichorium Intybus (Chicory) Root Oligosaccharides is the carbohydrate fraction isolated from the roots of Chicorium intybus.	
Galactoarabinan 9036-66-2	Galactoarabinan is the polysaccharide obtained from the extraction of one or more species of the larch tree, *Larix.* The structure of galactoarabinan is:[103]	
Ghatti Gum 9000-28-6	Ghatti Gum is the dried, gummy exudate obtained from the stems and bark of *Anogeissus latifolia.* Other sources: Ghatti gum has been defined as the dried exudate of *Anogeissus latifolia*.[28] Degradation studies have shown that ghatti gum is a polysaccharide that consists of a backbone of galactose units to which other sugars are attached.[104] The side chains can consist of arabinose residues and aldobiuronic acids.	
Glucomannan 37220-17-0 11078-31-2 76081-94-2	Glucomannan is the polymer of mannose containing side chains of glucose. Other sources: Glucomannan (a.k.a. konjac flour or konjac mannan) is a β-D-(1→4)-linked linear copolymer of glucose and mannose substituted with *O*-acetate every 9-19 sugar units.[105] It is derived from the tubers of *Amorphophallus* konjac. Due to the ß-glycosidic linkages between the glucose and mannose building blocks (β-1→4 linkages in the main chain and β-1→3 linkages at the branch points), glucomannan is commonly regarded as a non-digestible polysaccharide. Additionally, glucomannan contains acetyl groups, approximately one acetyl group per 19 sugar residues.[106]	

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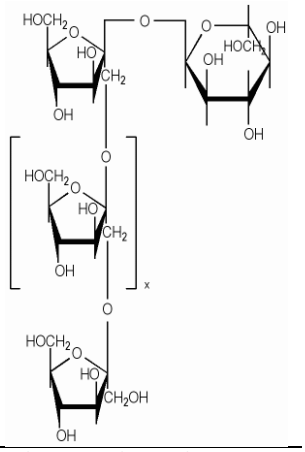
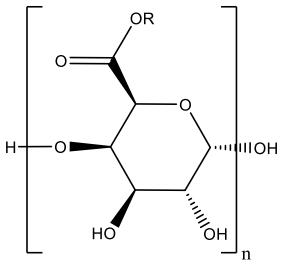
Ingredient CAS No.	Definition	Formula/structure
Inulin 9005-80-5	Inulin is the polysaccharide that conforms to the formula below. Other sources: Inulin has been identified as a fructan, a general term that is used to refer to naturally occurring plant oligo- and polysaccharides.[107] The term refers to any carbohydrate (linear or branched) in which one or more fructosyl-fructose links constitute the majority of the glycosidic bonds. Within the inulin-type fructans are two general groups of materials, inulin and its subsets, including oligofructose and fructooligosaccharides (FOS). FOS always terminate with a glucose molecule. Oligofructose most often contains only fructose molecules, but may end with a glucose molecule. Inulin is a polydisperse carbohydrate consisting mainly of β(2→1) fructosyl-fructose links and contains both GF_n and F_m compounds. The *n* or *m* represents the number of fructose units (F) linked to each other, which can vary from 2 to 70 with one terminal glucose (G). The terms oligofructose and FOS refer to inulin-type fructans with a maximum average degree of polymerization (DP) less than 10. Additionally, total hydrolysis of inulin yields fructose and glucose.[107]	
Pectin 9000-69-5	Pectin is a purified carbohydrate product obtained from the dilute acid extract of the inner portion of the rind of citrus fruits or from apple pomace. It consists chiefly of partially methoxylated polygalacturonic acids.	 where R is hydrogen or methyl
Phaseolus Angularis Seed Starch	Phaseolus Angularis Seed Starch is a starch obtained from the bean, *Phaseolus angularis.*	
Phaseolus Radiatus Seed Starch	Phaseolus Radiatus Seed Starch is the starch obtained from the seeds of the bean, *Phaseolus radiatus.*	
Pisum Sativum (Pea) Starch	Pisum Sativum (Pea) Starch is a starch obtained from *Pisum sativum.*	
Pueraria Lobata Starch 9005-25-8 (generic)	Pueraria Lobata Starch is the starch obtained from the roots of *Pueraria lobota.*	
Solanum Tuberosum (Potato) Starch 9005-25-8 (generic)	Solanum Tuberosum (Potato) Starch is a polysaccharide obtained from the potato, *Solanum tuberosum.*	

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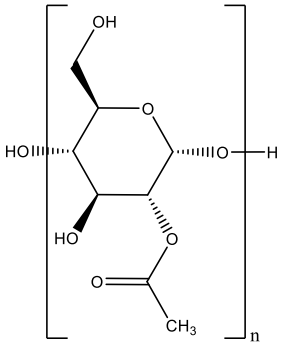
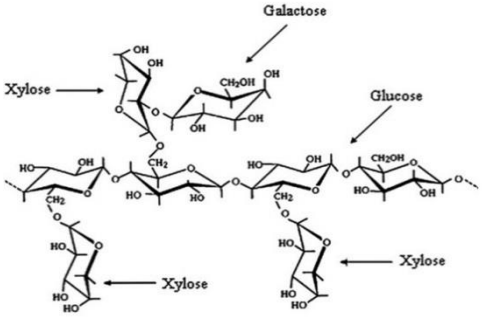
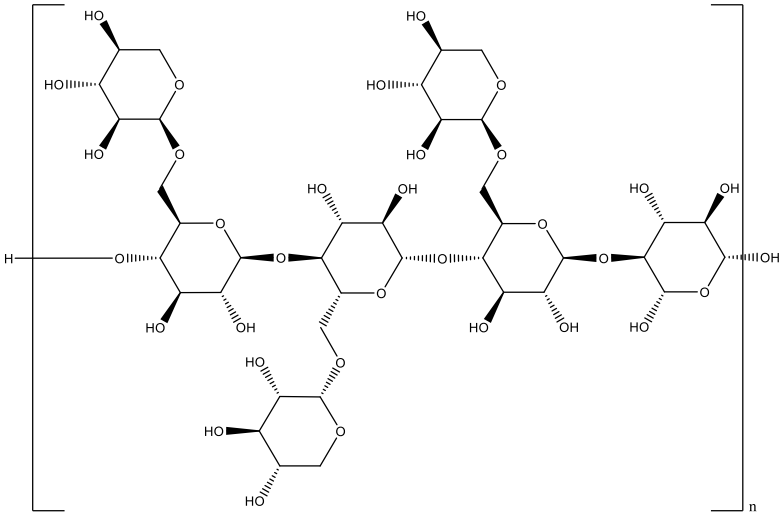
Ingredient CAS No.	Definition	Formula/structure
Starch Acetate 9045-28-7	Starch Acetate is the product obtained by the reaction of acetic acid with starch.	
Sterculia Urens Gum 9000-36-6 [VCRP name: Karaya Gum]	Sterculia Urens Gum is a dried exudate from the tree, *Sterculia urens.* Other source: Sterculia urens gum (a.k.a. karaya gum), the dried exudate of *Sterculia wens* Roxb. and other *Sterculia* spp. (fam. *Sterculiaceae),* is a complex, partially acetylated polysaccharide with a very high molecular weight.[39] Karaya gum is composed of the sugars galactose, rhamnose, and galacturonic acid.	
Tamarindus Indica Seed Gum 39386-78-2	Tamarindus Indica Seed Gum is the gum obtained from the seeds of *Tamarindus indica*.	
Tapioca Starch 9005-25-8	Tapioca Starch is the starch obtained from the roots of *Manihot esculenta.* It consists primarily of amylose and amylopectin.	
Triticum Vulgare (Wheat) Starch 9005-25-8 (generic)	Triticum Vulgare (Wheat) Starch is a starch obtained from wheat, *Triticum vulgare.*	
Xyloglucan 37294-28-3	Xyloglucan is an oligosaccharide containing a 1,4-β-glucan backbone with 1,6-α-xylosyl residues attached to the 6-position of β-glucosyl residues. Other source: The xyloglucan derived from tamarind seeds is composed of a (1-4)-β-glucan backbone chain, which has (1-6)-α-D-xylose branches that are partially substituted with (1-2)-β-D-galactoxylose.[108]	
***Branched – modified* (i.e., added sidechains are larger than acetate)**		
Calcium Starch Isododecenylsuccinate 194810-88-3	Calcium Starch Isododecenylsuccinate is the calcium salt of the product formed by the reaction of starch with isododecenylsuccinic anhydride.	

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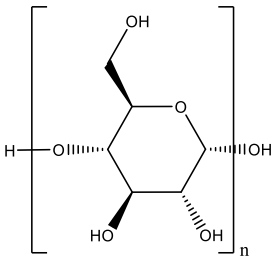
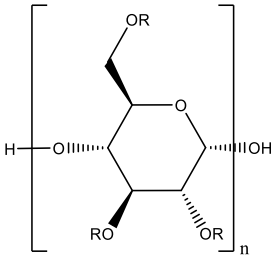
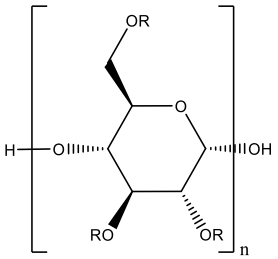
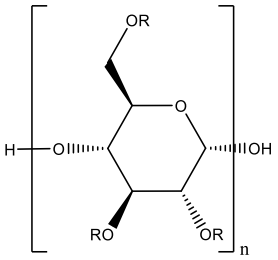
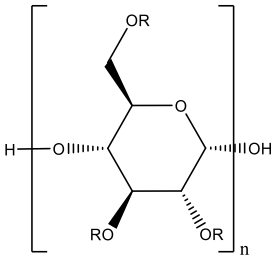
Ingredient CAS No.	Definition	Formula/structure
Calcium Starch Octenylsuccinate	Calcium Starch Octenylsuccinate is the calcium salt of the reaction product of octenylsuccinic anhydride with Zea Mays (Corn) Starch.	
Corn Starch Modified	Corn Starch Modified is the calcium salt of the ester formed from the reaction of 3-(dodecenyl)dihydro-2,5-furandione and corn starch in which the degree of substitution per glucose unit is less than 0.1.	
Dextrin 9004-53-9	Dextrin is a gum produced by the incomplete hydrolysis of starch.	
Dextrin Behenate 112444-74-3	Dextrin Behenate is the ester of Dextrin and Behenic Acid.	 wherein R is the residue of behenic acid
Dextrin Isostearate	Dextrin Isostearate is the ester of Dextrin and Isostearic Acid.	 wherein R is the residue of isostearic acid
Dextrin Laurate 79748-56-4	Dextrin Laurate is the ester of Dextrin and Lauric Acid.	 wherein R is the residue of lauric acid
Dextrin Myristate 93792-77-9	Dextrin Myristate is the ester of Dextrin and Myristic Acid.	 wherein R is the residue of myristic acid

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]

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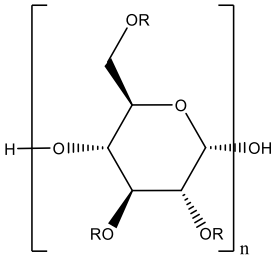
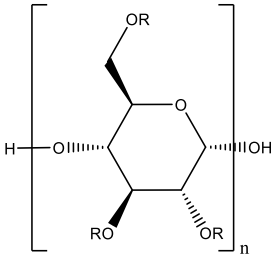
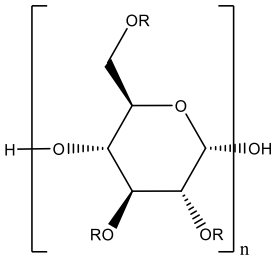
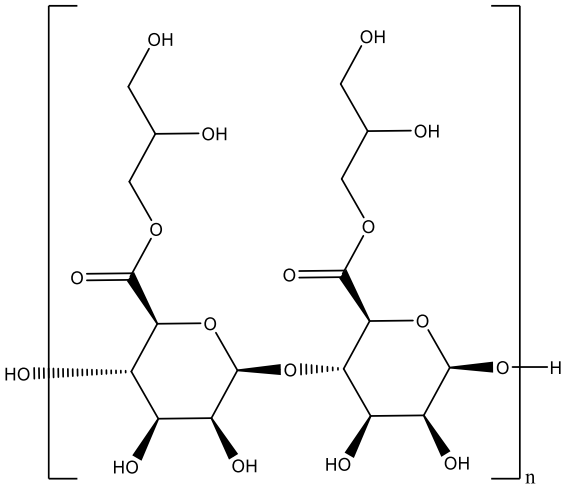
Ingredient CAS No.	Definition	Formula/structure
Dextrin Palmitate 83271-10-7	Dextrin Palmitate is the palmitic acid ester of Dextrin.	 wherein R is the residue of palmitic acid
Dextrin Palmitate/Ethylhexanoate 183387-52-2	Dextrin Palmitate/Ethylhexanoate is the mixed ester of Dextrin with palmitic and ethylhexanoic acids.	 wherein R is the residue of palmitic or ethylhexanoic acid
Dextrin Stearate 37307-33-8	Dextrin Stearate is the ester of Dextrin and Stearic Acid.	 wherein R is the residue of stearic acid
Glyceryl Alginate	Glyceryl Alginate is the ester of glycerin and Alginic Acid.	
Glyceryl Dimaltodextrin	Glyceryl Dimaltodextrin is the reaction product of Glycerin and Maltodextrin.	
Glyceryl Starch	Glyceryl Starch is a partially crosslinked corn starch.	

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]

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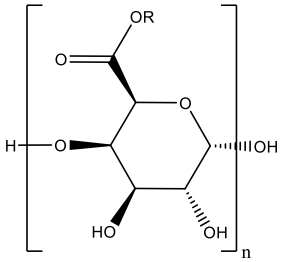
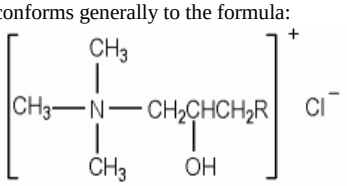
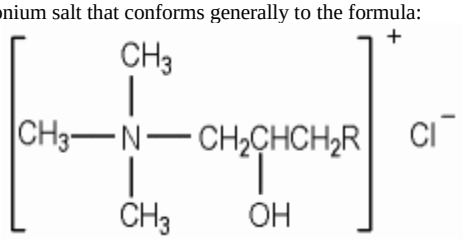
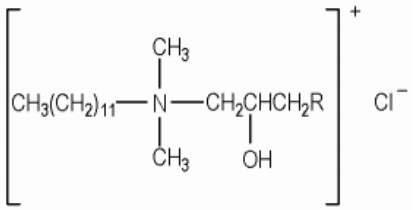
Ingredient CAS No.	Definition	Formula/structure
Hydrolyzed Pectin	Hydrolyzed Pectin is the hydrolysate of Pectin derived by acid, enzyme or other method of hydrolysis. *Pectin is a purified carbohydrate product obtained from the dilute acid extract of the inner portion of the rind of citrus fruits or from apple pomace. It consists chiefly of partially methoxylated polygalacturonic acids.*	 where R is hydrogen or methyl
Hydroxypropyltrimonium Hydrolyzed Corn Starch	Hydroxypropyltrimonium Hydrolyzed Corn Starch is the quaternary ammonium salt that conforms generally to the formula:	 where R represents the hydrolyzed corn starch moiety.
Hydroxypropyltrimonium Hydrolyzed Wheat Starch	Hydroxypropyltrimonium Hydrolyzed Wheat Starch is the quaternary ammonium salt that conforms generally to the formula:	 where R represents the hydrolyzed wheat starch moiety.
Hydroxypropyl Oxidized Starch	Hydroxypropyl Oxidized Starch is the reaction product of oxygen and Hydroxypropyl Starch.	
Hydroxypropyl Starch 68584-86-1 9049-76-7	Hydroxypropyl Starch is a propylene glycol ether of starch.	
Hydroxypropyltrimonium Maltodextrin Crosspolymer	Hydroxypropyltrimonium Maltodextrin Crosspolymer is a crosslinked polymeric quaternary ammonium salt prepared by the reaction of maltodextrin and glycidyltrimethylammonium chloride with epichlorohydrin.	
Laurdimonium Hydroxypropyl Hydrolyzed Wheat Starch	Laurdimonium Hydroxypropyl Hydrolyzed Wheat Starch is the quaternary ammonium chloride that conforms generally to the formula:	 where R represents the hydrolyzed wheat starch moiety.

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
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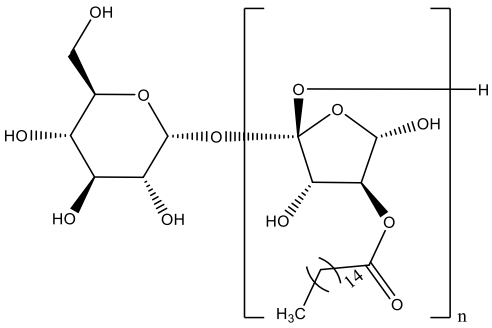
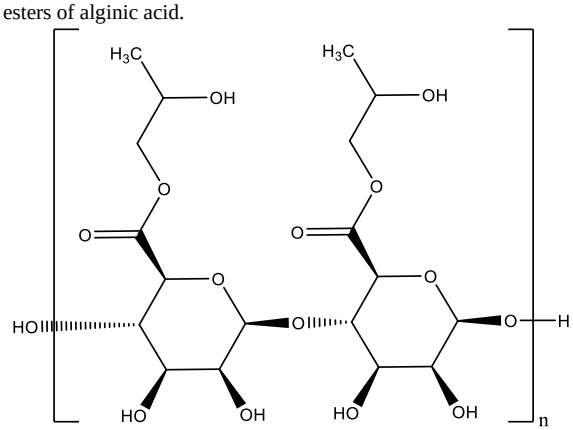
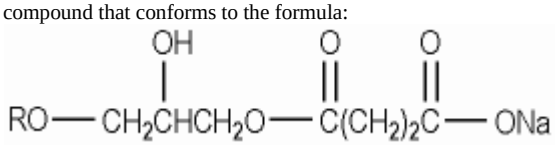
Ingredient CAS No.	Definition	Formula/structure
Palmitoyl Inulin	Palmitoyl Inulin is the condensation product of palmitic acid chloride and the carbohydrate, Inulin.	
Potassium Dextrin Octenylsuccinate	Potassium Dextrin Octenylsuccinate is the potassium salt of the reaction product of octenylsuccinic anhydride with Dextrin.	
Potassium Undecylenoyl Alginate	Potassium Undecylenoyl Alginate is the potassium salt of the condensation product of undecylenic acid chloride and Alginic Acid.	
Potassium Undecylenoyl Carrageenan	Potassium Undecylenoyl Carrageenan is the potassium salt of the condensation product of undecylenic acid chloride and Carrageenan.	
Potato Starch Modified	Potato Starch Modified is the ether formed from the reaction of haloethylaminodipropionic acid and potato starch in which the degree of substitution per glucose unit is less than 0.1.	
Propylene Glycol Alginate 9005-37-2	Propylene Glycol Alginate is a mixture of the propylene glycol esters of alginic acid.	
Sodium Carboxymethyl Inulin 430439-54-6	Sodium Carboxymethyl Inulin is the sodium salt of the product obtained by the reaction of chloroacetic acid with Inulin.	
Sodium Carboxymethyl Starch 9063-38-1	Sodium Carboxymethyl Starch is the sodium salt of a carboxymethyl derivative of starch.	
Sodium Dextrin Octenylsuccinate	Sodium Dextrin Octenylsuccinate is the sodium salt of the reaction product of octenylsuccinic anhydride with Dextrin.	
Sodium Hydrolyzed Potato Starch Dodecenylsuccinate	Sodium Hydrolyzed Potato Starch Dodecenylsuccinate is the sodium salt of the product obtained by the reaction of dextrin with dodecenylsuccinic anhydride.	
Sodium Hydroxypropyl Oxidized Starch Succinate	Sodium Hydroxypropyl Oxidized Starch Succinate is the organic compound that conforms to the formula:	 where R represents the oxidized starch moiety.
Sodium Oxidized Starch Acetate/Succinate	Sodium Oxidized Starch Acetate/Succinate is the sodium salt of product of the esterification of oxidized starch with acetic acid and succinic acid anhydrides.	

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
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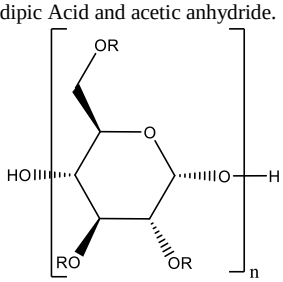
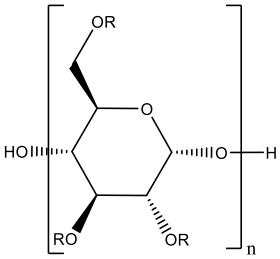
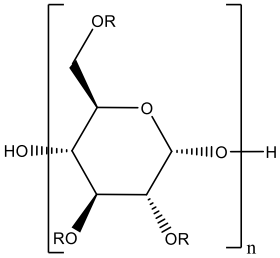
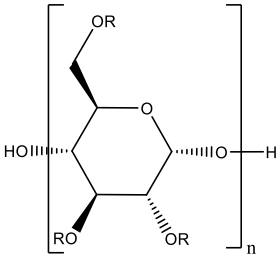
Ingredient CAS No.	Definition	Formula/structure
Sodium Starch Octenylsuccinate 52906-93-1 66829-29-6 70714-61-3	Sodium Starch Octenylsuccinate is the sodium salt of the reaction product of octenylsuccinic anhydride with Zea Mays (Corn) Starch.	
Sodium/TEA-Undecylenoyl Alginate	Sodium/TEA-Undecylenoyl Alginate is the mixed sodium and triethanolamine salt of the condensation product of undecylenic acid chloride and Alginic Acid.	
Sodium/TEA-Undecylenoyl Carrageenan	Sodium/TEA-Undecylenoyl Carrageenan is the mixed sodium and triethanolamine salt of the condensation product of undecylenic acid chloride and Carrageenan.	
Starch Acetate/Adipate 63798-35-6	Starch Acetate/Adipate is the product obtained by the reaction of Zea Mays (Corn) Starch with Adipic Acid and acetic anhydride.	 where R is adipate or acetate
Starch Diethylaminoethyl Ether 9041-94-5	Starch Diethylaminoethyl Ether is the product obtained by conversion of some hydroxyl groups in starch to diethylaminoethyl ether groups.	 where R is hydrogen or constitutes, with the attached oxygen, diethylaminoethyl ether
Starch Hydroxypropyltrimonium Chloride 56780-58-6	Starch Hydroxypropyltrimonium Chloride is the quaternary ammonium compound formed by the reaction of starch with 2,3-epoxypropyltrimethylammonium chloride. Other source: One of the starch hydroxypropyltrimonium chloride trade name materials is defiuned as an aqueous solution of a naturally derived cationic polysaccharide produced from food grade potato starch.[109]	 where R is hydrogen or constitutes, with the attached oxygen, hydroxypropyltrimonium
Starch Laurate	Starch Laurate is the product obtained by the reaction of lauric acid with starch.	 where R is hydrogen or laurate

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
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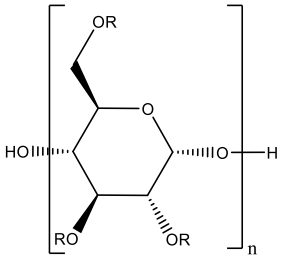
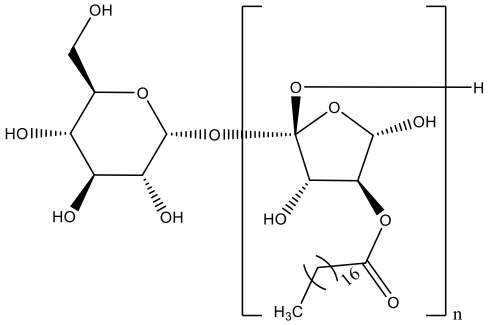
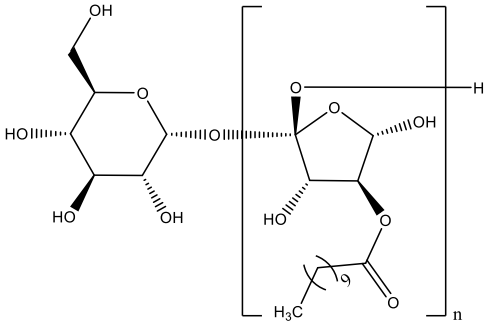
Ingredient CAS No.	Definition	Formula/structure
Starch Tallowate	Starch Tallowate is the ester of starch with the fatty acids derived from Tallow.	 where R is hydrogen or the residue of a fatty acid from tallow
Stearoyl Inulin	Stearoyl Inulin is the condensation product of stearic acid chloride with the carbohydrate, Inulin.	
Tapioca Starch Crosspolymer	Tapioca Starch Crosspolymer is Tapioca Starch crosslinked with epichlorohydrin.	
TEA-Dextrin Octenylsuccinate	TEA-Dextrin Octenylsuccinate is the triethanolamine salt of the reaction product of octenylsuccinic anhydride with Dextrin.	
Undecylenoyl Inulin	Undecylenoyl Inulin is the condensation product of undecylenic acid chloride with the carbohydrate, Inulin.	

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
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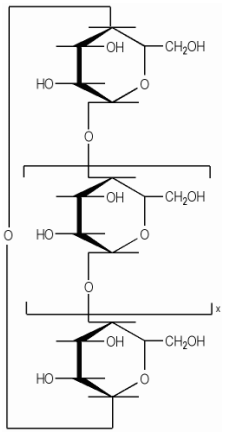
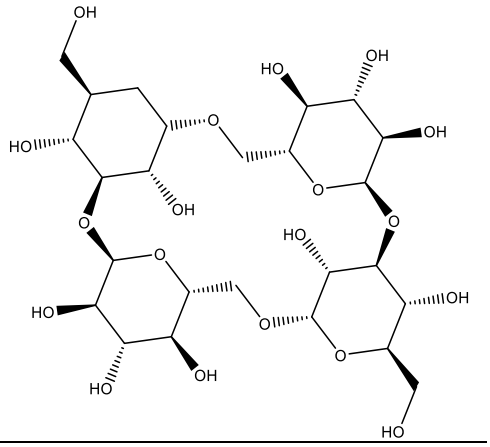
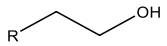
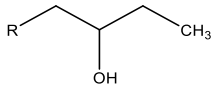
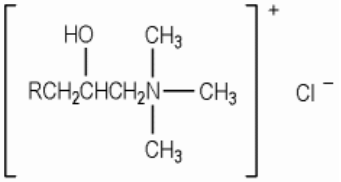
Ingredient CAS No.	Definition	Formula/structure
Cyclic		
Cyclodextrin 12619-70-4 7585-39-9	Cyclodextrin is a cyclic polysaccharide comprised of six to eight glucopyranose units. It conforms to the formula below: Other sources: Cyclodextrins are cyclic amylose-derived oligomers composed of a varying number of α-1-4-linked glucose units.[110] Cyclodextrins contain 6, 7, or 8 glucose units. β-Cyclodextrin is a carbohydrate consisting of seven glucose units.[111]	 where x may have values from 4 to 6.
Cyclotetraglucose 159640-28-5	Cyclotetraglucose is a cyclic polysaccharide comprised of four Glucose units.	
Cyclic - modified		
Hydroxyethyl Cyclodextrin	Hydroxyethyl Cyclodextrin is the hydroxyethyl ether of Cyclodextrin.	 where R represents the Cyclodextrin polymer.
Hydroxypropyl Cyclodextrin 128446-33-3 128446-35-5	Hydroxypropyl Cyclodextrin is a propylene glycol ether of Cyclodextrin.	 where R represents the Cyclodextrin polymer.
Cyclodextrin Hydroxypropyltrimonium Chloride	Cyclodextrin Hydroxypropyltrimonium Chloride is the organic compound that conforms to the formula:	 where R represents the Cyclodextrin polymer.

Table 1. Names, CAS Registry Numbers, Definitions and Idealized Structures of the Polysaccharide Gums.[1]
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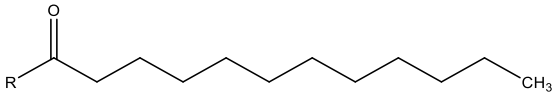
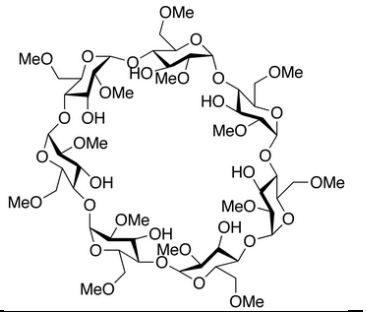
Ingredient CAS No.	Definition	Formula/structure
Cyclodextrin Laurate	Cyclodextrin Laurate is the product obtained by the reaction of Cyclodextrin and lauric acid chloride.	 where R represents the Cyclodextrin polymer.
Methyl Cyclodextrin 128446-36-6	Methyl Cyclodextrin is the product obtained by the methylation of Cyclodextrin.	
Unknown structural configuration		
Algae Exopolysaccharides	Algae Exopolysaccharides (Retired) are exopolysaccharides released by the fermentation of various species of microalgae of the divisions, Rhodophyta and Chlorophyta. The INCI Name, Algae Exopolysaccharides, originally published in 2010, was designated with a retired status in 2015. For an interim period of time, trade name assignments formerly published with the INCI Name Algae Exopolysaccharides will be retained in the retired monograph, and also published with the new name assignment based on the current genus and species name for the specific alga. For further information, consult the Introduction, Retired INCI Names.	
Cassia Angustifolia Seed Polysaccharide	Cassia Angustifolia Seed Polysaccharide is the polysaccharide fraction derived from the seed of Cassia angustifolia. Other source: Cassia angustofolia seed polysaccharide has been defined as a water-soluble galactomannan, consisting of D-galactose and D-mannose in the molar ratio of 3:2, isolated from the seeds of *Cassia angustifolia*.[112]	
Prunus Persica (Peach) Gum	Prunus Persica (Peach) Gum is the dried, gummy exudate obtained from *Prunus persica.*	
Unknown structural configuration - modified		
Hydrogenated Potato Starch 68412-29-3 (generic)	Hydrogenated Potato Starch is the end product of the controlled hydrogenation of Solanum Tuberosum (Potato) Starch.	
Hydrogenated Starch Hydrolysate 68425-17-2	Hydrogenated Starch Hydrolysate is the end-product of the controlled hydrogenation of hydrolyzed starch.	
Hydrolyzed Corn Starch Hydroxyethyl Ether	Hydrolyzed Corn Starch Hydroxyethyl Ether is the hydroxyethyl ether of Hydrolyzed Corn Starch.	
Hydrolyzed Corn Starch Octenylsuccinate 125109-81-1	Hydrolyzed Corn Starch Octenylsuccinate is the reaction product of octenylsuccinic anhydride with Hydrolyzed Corn Starch.	
Hydrolyzed Soy Starch 68412-29-3 (generic)	Hydrolyzed Soy Starch is the hydrolysate of soy starch derived by acid, enzyme or other method of hydrolysis.	
Hydrolyzed Starch 34612-38-9 68412-29-3 (generic)	Hydrolyzed Starch is the hydrolysate of starch obtained from *Ipomoea batatas*, *Manihot esculenta*, *Solanum tuberosum* or *Zea mays* by acid enzyme or other method of hydrolysis.	
Hydrolyzed Triticum Spelta Starch	Hydrolyzed Triticum Spelta Starch is the hydrolysate of the starch obtained from the grain, *Triticum spelta* derived by acid, enzyme or other method of hydrolysis.	
Hydrolyzed Wheat Starch 68412-29-3 (generic)	Hydrolyzed Wheat Starch is the hydrolysate of wheat starch derived by acid, enzyme or other method of hydrolysis.	

Table 2. Ingredient Functions in Cosmetic Products.[1]

Linear polysaccahrides and their salts	
Agar	Binders; Fragrance Ingredients; Viscosity Increasing Agents - Aqueous
Agarose	Skin-Conditioning Agents - Humectant; Viscosity Increasing Agents - Aqueous
Algin	Binders; Fragrance Ingredients; Viscosity Increasing Agents - Aqueous
Alginic Acid	Binders; Skin-Conditioning Agents - Miscellaneous; Viscosity Increasing Agents - Aqueous
Ammonium Alginate	Binders; Emulsion Stabilizers; Film Formers; Viscosity Increasing Agents - Aqueous
Amylose	Skin-Conditioning Agents - Humectant
Astragalus Gummifer Gum	Adhesives; Binders; Emulsion Stabilizers; Film Formers; Fragrance Ingredients; Viscosity Increasing Agents - Aqueous
Calcium Alginate	Fragrance Ingredients; Viscosity Increasing Agents - Aqueous
Calcium Carageenan	Emulsion Stabilizers; Film Formers; Viscosity Increasing Agents - Aqueous
Carrageenan	Binders; Fragrance Ingredients; Hair Conditioning Agents; Viscosity Increasing Agents - Aqueous
Magnesium Alginate	Binders; Emulsion Stabilizers; Viscosity Increasing Agents - Aqueous
Mannan	Film Formers; Viscosity Increasing Agents - Aqueous
Polianthes Tuberosa Polysaccharide	Skin-Conditioning Agents - Miscellaneous
Potassium Alginate	Binders; Emulsion Stabilizers; Viscosity Increasing Agents - Aqueous
Potassium Carrageenan	Binders; Emulsion Stabilizers; Film Formers; Viscosity Increasing Agents - Aqueous
Sodium Carrageenan	Binders; Emulsion Stabilizers; Film Formers; Viscosity Increasing Agents - Aqueous
TEA-Alginate	Binders; Emulsion Stabilizers; Viscosity Increasing Agents - Aqueous
Linear - modified	
Amylodextrin	Absorbents; Bulking Agents
Hydrolyzed Carrageenan	Skin-Conditioning Agents - Miscellaneous
Hydrolyzed Furcellaran	Skin Protectants
Maltodextrin	Absorbents; Binders; Dispersing Agents - Nonsurfactant; Emulsion Stabilizers; Film Formers; Hair Conditioning Agents; Skin-Conditioning Agents - Miscellaneous
Sodium Algin Sulfate	Skin-Conditioning Agents - Humectant
Branched – unmodified	
Amylopectin	Binders; Viscosity Increasing Agents - Aqueous
Aphanothece Sacrum Polysaccharide	Absorbents; Emulsion Stabilizers; Film Formers; Viscosity Increasing Agents - Aqueous
Arabinoxylan	Film Formers
Avena Sativa (Oat) Starch	Absorbents
Cassia Angustifolia Seed Polysaccharide	Skin-Conditioning Agents - Emollient
Cichorium Intybus (Chicory) Root Oligosaccharides	Skin-Conditioning Agents - Miscellaneous
Galactoarabinan	Film Formers; Fragrance Ingredients
Ghatti Gum	Binders; Emulsion Stabilizers; Surfactants - Emulsifying Agents; Viscosity Increasing Agents - Aqueous
Glucomannan	Skin Protectants; Skin-Conditioning Agents - Miscellaneous
Inulin	Skin-Conditioning Agents - Humectant
Pectin	Binders; Emulsion Stabilizers; Oral Health Care Drugs; Viscosity Increasing Agents - Aqueous
Phaseolus Angularis Seed Starch	Absorbents
Phaseolus Radiatus Seed Starch	Abrasives; Bulking Agents
Pisum Sativum (Pea) Starch	Absorbents; Opacifying Agents; Slip Modifiers

Table 2. Ingredient Functions in Cosmetic Products.[1]

Pueraria Lobata Starch	Absorbents; Opacifying Agents; Slip Modifiers
Solanum Tuberosum (Potato) Starch	Absorbents; Binders; Bulking Agents; Viscosity Increasing Agents - Aqueous
Starch Acetate	Hair Conditioning Agents; Skin-Conditioning Agents - Emollient
Sterculia Urens Gum	Adhesives; Binders; Emulsion Stabilizers; Fragrance Ingredients; Hair Fixatives; Viscosity Increasing Agents - Aqueous
Tamarindus Indica Seed Gum	Adhesives; Emulsion Stabilizers; Skin-Conditioning Agents - Humectant; Viscosity Increasing Agents - Aqueous
Tapioca Starch	Viscosity Increasing Agents - Aqueous
Triticum Vulgare (Wheat) Starch	Abrasives; Absorbents; Binders; Bulking Agents; Viscosity Increasing Agents - Aqueous
Xyloglucan	Humectants
Branched – modified **(i.e., added sidechains are larger than acetate)**	
Calcium Starch Isododecenylsuccinate	Absorbents; Skin-Conditioning Agents - Emollient
Calcium Starch Octenylsuccinate	Absorbents; Emulsion Stabilizers; Viscosity Increasing Agents - Aqueous
Corn Starch Modified	Absorbents; Film Formers; Skin-Conditioning Agents - Miscellaneous; Viscosity Increasing Agents - Nonaqueous
Dextrin	Absorbents; Binders; Bulking Agents; Viscosity Increasing Agents - Aqueous
Dextrin Behenate	Anticaking Agents; Surfactants - Emulsifying Agents
Dextrin Isostearate	Skin-Conditioning Agents - Miscellaneous
Dextrin Laurate	Anticaking Agents; Surfactants - Emulsifying Agents
Dextrin Myristate	Anticaking Agents; Surfactants - Emulsifying Agents
Dextrin Palmitate	Anticaking Agents; Surfactants - Emulsifying Agents
Dextrin Palmitate/Ethylhexanoate	Anticaking Agents; Surfactants - Emulsifying Agents
Dextrin Stearate	Anticaking Agents; Surfactants - Emulsifying Agents
Glyceryl Alginate	Skin-Conditioning Agents - Emollient; Viscosity Increasing Agents - Aqueous
Glyceryl Dimaltodextrin	Humectants; Skin-Conditioning Agents - Humectant
Glyceryl Starch	Absorbents; Binders
Hydrolyzed Pectin	Skin-Conditioning Agents - Miscellaneous
Hydroxypropyltrimonium Hydrolyzed Corn Starch	Antistatic Agents; Film Formers; Hair Conditioning Agents; Hair Fixatives; Hair-Waving/Straightening Agents
Hydroxypropyltrimonium Hydrolyzed Wheat Starch	Antistatic Agents; Hair Conditioning Agents
Hydroxypropyl Oxidized Starch	Film Formers
Hydroxypropyl Starch	Dispersing Agents - Nonsurfactant; Viscosity Increasing Agents - Aqueous
Hydroxypropyltrimonium Maltodextrin Crosspolymer	Dispersing Agents - Nonsurfactant
Laurdimonium Hydroxypropyl Hydrolyzed Wheat Starch	Antistatic Agents; Hair Conditioning Agents
Palmitoyl Inulin	Skin-Conditioning Agents - Emollient; Surfactants - Emulsifying Agents
Potassium Dextrin Octenylsuccinate	Emulsion Stabilizers; Hair Conditioning Agents; Humectants; Skin-Conditioning Agents - Emollient; Surfactants - Emulsifying Agents
Potassium Undecylenoyl Alginate	Emulsion Stabilizers; Hair Conditioning Agents; Skin-Conditioning Agents - Miscellaneous
Potassium Undecylenoyl Carrageenan	Emulsion Stabilizers; Hair Conditioning Agents; Skin-Conditioning Agents - Miscellaneous
Potato Starch Modified	Viscosity Increasing Agents - Aqueous
Propylene Glycol Alginate	Binders; Fragrance Ingredients; Viscosity Increasing Agents - Aqueous
Sodium Carboxymethyl Inulin	Chelating Agents; Viscosity Increasing Agents - Aqueous
Sodium Carboxymethyl Starch	Binders; Emulsion Stabilizers; Film Formers; Viscosity Increasing Agents - Aqueous

Table 2. Ingredient Functions in Cosmetic Products.[1]

Sodium Dextrin Octenylsuccinate	Emulsion Stabilizers; Hair Conditioning Agents; Humectants; Skin-Conditioning Agents - Emollient; Surfactants - Emulsifying Agents
Sodium Hydrolyzed Potato Starch Dodecenylsuccinate	Surfactants – Foam Boosters
Sodium Hydroxypropyl Oxidized Starch Succinate	Film Formers; Hair Conditioning Agents; Humectants; Skin-Conditioning Agents - Miscellaneous
Sodium Oxidized Starch Acetate/Succinate	Film Formers; Hair Conditioning Agents; Humectants; Skin-Conditioning Agents - Miscellaneous
Sodium Starch Octenylsuccinate	Absorbents; Emulsion Stabilizers; Viscosity Increasing Agents - Aqueous
Sodium/TEA-Undecylenoyl Alginate	Emulsion Stabilizers; Hair Conditioning Agents; Skin-Conditioning Agents - Miscellaneous
Sodium/TEA-Undecylenoyl Carrageenan	Emulsion Stabilizers; Hair Conditioning Agents; Skin-Conditioning Agents - Miscellaneous
Starch Acetate/Adipate	Viscosity Increasing Agents - Aqueous
Starch Diethylaminoethyl Ether	Film Formers; Skin-Conditioning Agents - Miscellaneous
Starch Hydroxypropyltrimonium Chloride	Antistatic Agents; Dispersing Agents - Nonsurfactant; Emulsion Stabilizers; Hair Conditioning Agents; Viscosity Increasing Agents - Aqueous
Starch Laurate	Abrasives
Starch Tallowate	Skin-Conditioning Agents - Emollient
Stearoyl Inulin	Skin-Conditioning Agents - Emollient; Surfactants - Emulsifying Agents
Tapioca Starch Crosspolymer	Absorbents; Binders
TEA-Dextrin Octenylsuccinate	Emulsion Stabilizers; Hair Conditioning Agents; Humectants; Skin-Conditioning Agents - Emollient; Surfactants - Emulsifying Agents
Undecylenoyl Inulin	Emulsion Stabilizers; Skin-Conditioning Agents - Emollient
Cyclic	
Cyclodextrin	Absorbents; Chelating Agents
Cyclotetraglucose	Binders; Bulking Agents; Skin-Conditioning Agents - Humectant; Viscosity Increasing Agents - Aqueous
Cyclic - modified	
Hydroxyethyl Cyclodextrin	Skin-Conditioning Agents - Miscellaneous
Hydroxypropyl Cyclodextrin	Chelating Agents; Emulsion Stabilizers
Cyclodextrin Hydroxypropyltrimonium Chloride	Film Formers; Skin-Conditioning Agents - Humectant; Viscosity Increasing Agents - Aqueous
Cyclodextrin Laurate	Film Formers; Skin Protectants; Skin-Conditioning Agents - Humectant
Methyl Cyclodextrin	Chelating Agents
Unknown structural configuration	
Algae Exopolysaccharides	Film Formers; Skin Protectants; Skin-Conditioning Agents - Humectant; Slip Modifiers
Prunus Persica (Peach) Gum	Viscosity Increasing Agents - Aqueous
Unknown structural configuration - modified	
Hydrogenated Potato Starch	Viscosity Increasing Agents - Aqueous
Hydrogenated Starch Hydrolysate	Film Formers; Humectants; Oral Care Agents; Skin-Conditioning Agents - Humectant
Hydrolyzed Corn Starch Hydroxyethyl Ether	Emulsion Stabilizers; Humectants; Skin-Conditioning Agents - Humectant; Viscosity Increasing Agents - Aqueous
Hydrolyzed Corn Starch Octenylsuccinate	Absorbents; Binders; Film Formers
Hydrolyzed Soy Starch	Skin-Conditioning Agents - Miscellaneous
Hydrolyzed Starch	Humectants; Skin Protectants; Skin-Conditioning Agents - Humectant
Hydrolyzed Triticum Spelta Starch	Skin-Conditioning Agents - Miscellaneous
Hydrolyzed Wheat Starch	Skin-Conditioning Agents - Humectant

Table 3. Properties and Method of Manufacture of Polysaccharide Gums

Linear Polysaccharides and Their Salts

Carrageenan

Average Molecular Weight: > 100,000 Da.[35] **Molecular Weight Range:** 196,000–257,000 Da.[113]

Stability: Data on carrageenans (in their sodium ion form without co-gelling cations) included κ-carrageenan from *Euchema cottoni*i, ι-carrageenan from *Eucheuma spinosoum*, a κ/λ mixture extracted from Chondrus crispus, and a κ/λ hybrid carrageenan from *Gigartina radula*. Reasonable stability to heating at 75˚C down to pH 4, and the rate of depolymerization increases dramatically as the pH decreases from 4 to 3. ι-Carrageenan is the most stable form, while κ-carrageenan has the greatest susceptibility to acid hydrolysis. The carrageenans from *Gigartina radula* and *Chondrus crispus* have intermediate stability.[114]

Carrageenan in the presence of co-gelling cations is much more stable than carrageenan in sodium ion form at 37˚C. However, at higher temperatures, the carrageenan is in the random coil state and is more susceptible to acid degradation. Studies of the stability of κ-carrageenan in the presence of potassium ions have shown that acid-catalyzed hydrolysis occurs at temperatures between 55˚C and 95˚C. Degradation was described as a first-order random hydrolysis process. A 25% reduction in molecular weight was produced at pH 3 after 1.4 h at 50˚C, and after only 28 seconds at 90˚C. At pH 4, a similar reduction in molecular weight was recorded after 8 h at 50˚C and after 15 minutes at 90˚C.[114]

Inulin

Method of Manufacture: Extraction from the roots of *Cichorium intybus*.[115]

Linear - Modified

Amylodextrin

Method of Manufacture: Prepared from waxy maize by enzymatic hydrolysis with pullulanase.[116]

Hydrolyzed Furcellaran

Method of Manufacture: The polymer furcellaran (a carrageenan [Kappa type]) obtained from *Furcellaria lumbricallis* is depolymerized by sub-critical CO_2 with a low percentage of water, and the product is an opalescent liquid **(See Figure 2)**.[73]

Maltodextrin

Method of Manufacture: Prepared as a white powder or concentrated solution by partial hydrolysis of corn starch, potato starch, or rice starch with suitable acids and enzymes.[117]

Branched - Unmodified

Arabinoxylan

Molecular Weight: 65 to 66 kDa (obtained by sedimentation),[118] 800 - 5000 kDa (obtained by gel filtration),[119] and 70 - 1,000 kDa (obtained by gel filtration).[120]

Cichorium Intybus (Chicory) Root Oligosaccharides

Method of Manufacture: Extraction from the roots of Cichorium intybus.[115]

Ghatti Gum

Molecular Weight: ≈ 8.94 x 10^7 Da.[121]

Glucomannan

Average Molecular Weight: 1,000,000 Da; between 200,000 and 2,000,000 Da (commercial samples).[122]

Form: biphasic liquid crystal phase in water at 7 weight% concentration; becomes completely anisotropic at >10 weight%.[106]

Decomposition: Begins to decompose at approximately 250˚C; decomposition is complete at 350˚C.[122]

Method of Manufacture: Obtained by a dry milling process of thin tuber (*Amorphophallus konjac*) slices.[105] Can also be obtained from monocot storage organs other than tubers, such as leaves, bulbs, roots, or seeds.[122] Glucomannan is found in specific large-sized idioblast cells located in the protoplast, and raphide crystal bundles of oxalic acid are enveloped in the polysaccharide. During processing, focus is placed on eliminating the protein membrane of these cells and removing the needle-shaped oxalic acid crystals by sieving, to give residual levels of approximately 0.2% for crude powder and lower for refined grades.[122]

Branched - Modified

Carboxymethyl Inulin

Method of Manufacture: Synthesized by reacting inulin with the sodium salt of monochloroacetic acid in the presence of sodium hydroxide.[123]

Table 3. Properties and Method of Manufacture of Polysaccharide Gums

Corn Starch Modified

Method of Manufacture: aqueous corn starch slurry reaction with 3-(dodecenyl) dihydro-2,5-furandione.[66,124]

Dextrin

Method of Manufacture: Dilute acid (e.g. HNO3) is added to native starch, and the starch is pre-dried. Next, pre-dried-starch is roasted at a temperature between 110°C and 150°C until the color of the starch changes to what is described as appropriate whiteness.[125] Another production method begins with the suspension of starch in water and adjustment of the pH to between 6 and 8. An enzyme (e.g., liquefying-type amylase) is added to the slurry, which is liquefied at 80°C and 90°C. Starch syrup is degraded to an appropriate viscosity, and the enzyme is made inactive. The syrup is purified by diatomite, active-carbon, ion-exchange resin and then dried.[125]

Dextrin Myristate

Form: Powder or particles.[67]

Color: White to pale yellow.[67]

Odor: Odorless or characteristic.[67]

Melting Point/Freezing Point: 50 ~ 150˚C.[67]

Flash Point: 210˚C.[67]

Solubility: Insoluble in water, methanol, and ethanol; soluble in xylene, benzene, chloroform, and carbon tetrachloride.[67]

Method of Manufacture: An esterification reaction involving 3-methylpyridine (beta-picoline) and dimethylformamide (DMF) is followed by percolation, washing (methanol and water), centrifugation, drying, riddle, and use of magnets. Riddle is defined as a screening or sieving process that removes large particulate material. Magnets are used to remove metal particles.[126]

Dextrin Isostearate

Form: Soft solid.[127]

Color: Colorless to pale yellow.[127]

Odor: Odorless or characteristic.[127]

Melting Point/Freezing Point: 60 ~ 70°C.[127]

Flash Point: > 200°C.[127]

Solubility: Insoluble in water, methanol, and ethanol; soluble in xylene, benzene, chloroform, and carbon tetrachloride.[127]

Method of Manufacture: The method of manufacture for dextrin isostearate begins with an esterification reaction involving 3-methylpyridine (beta-picoline) and n-heptane, followed by percolation, washing (methanol), drying, and filtration.[128]

Dextrin Palmitate

Form: Powder or particles.[68,69]

Color: White to pale yellow.[68,69]

Odor: Odorless or characteristic.[68,69]

Melting Point/Freezing Point: 50 ~ 130˚C; 100 ~ 130˚C.[68,69]

Flash Point: 200 ~ 250˚C.[68,69]

Solubility: Insoluble in water, methanol, and ethanol; soluble in xylene, benzene, chloroform, and carbon tetrachloride.[68,69]

Method of Manufacture: An esterification reaction involving 3-methylpyridine (beta-picoline) and dimethylformamide (DMF) is followed by percolation, washing (methanol and water), centrifugation, drying, riddle, and use of magnets. Riddle is defined as a screening or sieving process that removes large particulate material. Magnets are used to remove metal particles.[126]

Dextrin Palmitate/Ethylhexanoate

Form: Powder or particles.[129]

Color: White to pale yellow.[129]

Odor: Odorless or characteristic.[129]

Melting Onset Temperature: 120°C.[129]

Flash Point: 216°C.[129]

Table 3. Properties and Method of Manufacture of Polysaccharide Gums

Dextrin Palmitate/Ethylhexanoate

Solubility: Insoluble in water, methanol, and ethanol; soluble in xylene, benzene, chloroform, and carbon tetrachloride.[129]

Method of Manufacture: An esterification reaction involving 3-methylpyridine (beta-picoline) and dimethylformamide (DMF) is followed by percolation, washing (methanol and water), centrifugation, drying, riddle, and use of magnets. Riddle is defined as a screening or sieving process that removes large particulate material. Magnets are used to remove metal particles.[126]

Glyceryl Dimaltodextrin

Method of Manufacture: Production of maltodextrins involves the obtention of products consisting of D-glucose units that are linked primarily by α(1→4) bonds and having dextrose equivalents less than 20.[130]

Hydroxypropyl Starch

Method of Manufacture: Sodium sulfate (Na2SO4) and sodium hydroxide (NaOH) are dissolved in water, and starch and propylene oxide are added, and heated to 38°C to 42°C. After the reaction is finished, the slurry is neutralized by acid (H2SO4). The starch is then dewatered, washed, and dried. The slurry of hydroxyl-propyl starch may also be degraded by an enzyme (e.g., liquefying-type amylase), purified by diatomite and active-carbon, and then dried.[125]

Potato Starch Modified

Method of Manufacture: An aqueous potato starch slurry is reacted with haloethylaminopropionic acid. This reaction is followed by washing, filtration, and drying.[70]

Sodium Dextrin Octenylsuccinate

Method of Manufacture: Method 1: The slurry of sodium starch octenylsuccinate is degraded by an enzyme (e.g., liquefying-type amylase), purified by diatomite and active-carbon, and dried. The dried starch film is crushed into a fine powder. **Method 2:** Dextrin solution and octenylsuccinic anhydride are esterified, whereby the pH value is adjusted between 7 and 8 with alkaline (triethanolamine; sodium hydroxide solution, potassium hydroxide solution). The sodium dextrin octenylsuccinate manufactured according to this method is sold as a liquid. **Method 3:** Dextrin solution and octenylsuccinic anhydride are esterified, whereby the pH value is adjusted between 7 and 8 with sodium hydroxide solution. The solution is then dried.[125]

Sodium Hydrolyzed Potato Starch Dodecenylsuccinate

Solubility: Soluble in water (149.5 - 158.2 g/l).[131]

Method of Manufacture: Reaction of a hydrolyzed starch with dodecenylsuccinic anhydride.[132]

Sodium Hydroxypropyl Oxidized Starch Succinate

Method of Manufacture: Native starch (CAS No. 9005-25-8) and oxidized starch (CAS No. 065996-62-5) can be modified by reacting starch with etherifying and/or esterifying reagents in the presence of an alkaline catalyst.[15,133]

Reaction to form 2-hydroxypropyl, oxidized starch succinate

Starch 2-Hydroxypropyl Ether, Oxidized + Succinic Anhydride → Starch, 2-Hydroxypropyl, Oxidized, Succinic Acid Ester

$$\text{ST-O-CH}_2\text{-CH-CH}_3\text{OH} \qquad \text{C}_4\text{H}_4\text{O}_3\text{CH}_2 \qquad \text{ST-O-(C-CH}_3)_x\text{-(C}_4\text{H}_4\text{O)}_y$$

$$\text{O} \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \text{O}$$

Sodium Starch Octenylsuccinate

Method of Manufacture: Starch is suspended in water, and octenylsuccinic anhydride is added. The slurry is heated to approximately 40°C, and the pH value is adjusted between 6 and 9 with dilute sodium hydroxide solution. The pH value of the solution is stable between 7 and 8, and the slurry is neutralized by acid (H2SO4). The starch is then dewatered, washed, and dried. Sodium starch octenylsuccinate may also be suspended in water and dried. The dried starch film is crushed into a find powder.[125]

Starch Hydroxypropyltrimonium Chloride

Molecular Weight: 2,000,000 Da.[109]

Table 3. Properties and Method of Manufacture of Polysaccharide Gums

Starch Hydroxypropyltrimonium Chloride

Form: Clear to slightly hazy liquid (clear in 1:5 water solution).[109]

Dry Substance (%) 31-33.[109]

Color, Gardner: ≤ 2.5.[109]

Odor: Very mild; slightly sweet.[109]

pH @ 20°C: 3.5-4.5.[109]

Method of Manufacture: The starting materials for the production of starch hydroxypropyltrimonium chloride are: oxidized starch and the cationic reagent 3-chloro-2-hydroxypropyltrimethylammonium chloride (CAS No. 3327-22-8).[133] The reaction to form cationic starch ether appears below:[133]

Starch + 2,3-Epoxypropyltrimethylammonium Chloride → Starch Hydroxypropyl Trimethylammonium Chloride

ST-OH CH_2-CH-CH_2-$N^+(CH_3)_3$ Cl ST-O-CH_2-CH-$CH_2N^+(CH_3)_3$-Cl

According to another source, starch hydroxypropyltrimonium chloride is produced by an aqueous starch slurry reaction with 2,3-epoxypropyltrimethylammonium chloride in the presence of isopropanol. This reaction is followed by washing with isopropanol/water, and the material is then filtered and dried.[134]

Stearoyl Inulin

Form: Powder or particles.[71,72]

Color: White to pale yellow.[71,72]

Odor: Odorless or characteristic.[71,72]

Melting Onset Temperature: 64˚C; 68.2˚C.[71,72]

Flash Point: 210˚C; 214˚C.[71,72]

Solubility: Insoluble in water, methanol, and ethanol.[71,72]

Cyclic

Cyclodextrin

Solubility: Low aqueous solubility (1.85 g/100mL, β-Cyclodextrin).[135]

Unknown Structural Configuration

Algae Exopolysaccharides

Method of Manufacture: Microalgae is grown in fermenters under conditions that promote the production of the exopolysaccharide, which is secreted by the microalgae. The exopolysaccharides are removed from the cells via filtration or centrifugation, followed by precipitation with alcohol. The exopolysaccharide is then dried and ground to a fine powder. The supplier of this information stated that the CAS number for the ingredient produced (algae exopolysaccharides) is 1122611-69-1, and that the empirical formula for this ingredient is (C27H44O27S)n. Additionally, it was noted that this is the CAS number for D-galactopyranose.[136]

Cassia Angustifolia Seed Polysaccharide

Average Molecular Weight: 9.66 x 10^4 Da.[137]

Unknown Structural Configuration - Modified

Hydrolyzed Starch

Method of Manufacture: Raw Material (Starch) →Starch slurry→Liquefaction by thermostable α-amylase→Saccharification by isoamylase (to debranch starch amylose) and exomaltotetraohydrolase (to produce maltotetraose)→Heat treatment (inactivation of enzymes)→Filtration→Concentration→Decoloration→Filtration→Storage →Filling and weighing→Hydrolyzed starch.[138,139]

Table 4. Composition/Impurities Data on Polysaccharide Gums

Linear Polysaccharides and Their Salts

Algin

After exhaustive methylation of alginic acid, reduction to the corresponding mannoside derivative, and hydrolysis, chromatographic separation indicated that the hydrolyzate contained 88% 2,3-dimethylmannose, 4.5% monomethylmannose, 1% 2,3,4-trimethylmannose, and 6% dimethylglucose.[87]

Carrageenan

The low-molecular-weight forms of carrageenan are <5% of the total composition of the commercial product.[35]

Twenty-nine samples of food-grade refined carrageenan were analyzed using high-performance liquid gel permeation chromatography. Each sample had no obvious peak of poligeenan (which is defined as degraded carrageenan, detection limit ≈ 5%).[140] Poligeenan is produced by a different manufacturing process of seaweed that involves intentional extensive acid hydrolysis, resulting in sulfated galactose polymers with a weight average molecular weight of ~ 15,000 Da.[35] Furthermore**,** according to another source, the molecular weight of poligeenan is in the range of 10,000 to 20,000 Da.[141]

Inulin

According to the *Food Chemicals Codex*, inulin should contain no more than the following: 1 mg/kg lead, 0.2% ash, and 15% (combined) of monosaccharides (as fructose and glucose) and disaccharides (as sucrose), calculated on the dried basis.[115]

Linear - Modified

Hydrolyzed Furcellaran (Mixtures).[73,142]

Mixture 1: Components: hydrolyzed furcellaran (0.6%), concentrate of sea water (0.05%), phenoxyethanol (1%), and water (98.35%). **Impurities:** contains heavy metals at a concentration < the limit of quantification, except for Cr (4.74 mg/kg), Ni (1.93 mg/kg), Pb (0.23 mg/kg), Co (0.17 mg/kg), and As (0.11 mg/kg); contains iodine at a concentration < the limit of quantification (i.e., 1 ppm; contains polychlorobiphenyl (PCB) at a concentration < the limit of quantification (i.e., 2 µg/kg) and research pesticides at a concentration < the limit of quantification (i.e., 10 ng/g).

Mixture 2: Components: hydrolyzed furcellaran (1.35%), phenoxyethanol (1%), and water (97.65%)

Mixture 3: Components: hydrolyzed furcellaran (1.90%), citric acid (0.05%), potassium sorbate (0.10%), and water 97.95%). **Impurities:** contains heavy metals at a concentration < the limit of quantification, except for Cr (0.162 mg/kg) and Pb (0.08 mg/kg); contains iodine at a concentration < the limit of quantification (i.e., 9 ppm); contains PCB at a concentration < the limit of quantification (i.e.,10 µg/kg) and research pesticides at a concentration < the limit of quantification (i.e., 10 ng/g).

Maltodextrin

According to the *Food Chemicals Codex*, maltodextrin should contain no more than the following: 0.5 mg/kg lead, 0.0025% sulfur dioxide, 1% maltodextrins produced from high-amylose starches, and 0.5% all other types of maltodextrins.[115]

Branched - Unmodified

Arabinoxylan

Arabinoxylans are complex, as the side branches of the main chain arabinose and xylose units contain small amounts of xylopyranose, galactopyranose, and α-D-glucuronic acid or 4-*O*-methyl-α-D-glucuronic acid.[143]

Glucomannan

Konjac flour consists of the following: carbohydrates (as water-soluble fiber, ~75% of glucomannan composition), protein (2-8%), fat (<1%), ash (3-5%), and moisture (<15%).[105]

Sterculia Urens Gum

Commercial sterculia urens gum contains 19%-21% of rhamnose and similar proportions of galactose and galacturonic acid.[36] Nitrogen content (probably non-protein in nature) of 0.07% has also been reported.[51]

Branched - Modified

Dextrin Myristate

Dextrin myristate contains: dextrin myristate (> 95%); moisture, based on loss of drying (< 1%); myristic acid (< 5%); 3-Methylpyridine (beta-picoline) (< 300 ppm); DMF (< 5 ppm, detection limit); and methanol (< 5 ppm, detection limit).[144]

Dextrin Palmitate

Dextrin palmitate contains: dextrin palmitate (> 95%); moisture, based on loss on drying (< 1%); palmitic acid (< 5%); 3-methylpyridine (beta-picoline) (< 300 ppm; < 1,000 ppm); DMF (< 5 ppm, detection limit); and methanol (< 5 ppm, detection limit).[145,146]

Dextrin Palmitate/Ethylhexanoate

Dextrin Palmitate/Ethylhexanoate contains: dextrin palmitate/ethylhexanoate (> 95%); moisture, based on loss on drying (< 3%); palmitic acid and 2-ethylhexanoic acid (< 5%); 3-Methylpyridine (beta-picoline) (< 300 ppm); DMF (< 5 ppm, detection limit); and methanol (< 5 ppm).[147]

Table 4. Composition/Impurities Data on Polysaccharide Gums

Dextrin Isostearate

Dextrin isostearate contains: dextrin isostearate (> 95%); isostearic acid (< 5%); 3-methylpyridine (beta-picoline) (< 300 ppm); heptane (< 200 ppm); and methanol (< 5 ppm, detection limit).[148]

Sodium Hydrolyzed Potato Starch Dodecenylsuccinate

Impurities: antimony (7.53 mg/kg), arsenic (< 2 mg/kg), barium (0.271 mg/kg), cadmium (< 0.2 mg/kg), chromium (< 0.25 mg/kg), cobalt (< 1.5 mg/kg), copper (<0.25 mg/kg), lead (< 1.5 mg/kg), nickel (< 1 mg/kg), selenium (< 4.86 mg/kg), zinc (1.49 mg/kg), and mercury (< 0.1 mg/kg).[149]

Starch Hydroxypropyltrimonium Chloride

Starch hydroxypropyltrimonium chloride consists of approximately 30% solids, and is preserved with food grade sodium benzoate.[109]

Impurities/residuals data: diol levels (< 2%), enol levels (< 1.5%), and quaternizing agent (< 0.1%).[134]

Stearoyl Inulin

Stearoyl inulin contains: stearoyl inulin (> 95%); moisture, based on loss on drying (< 1%); stearic acid (< 5%); 3-Methylpyridine (beta-picoline) (< 300 ppm); DMF (< 5 ppm, detection limit); and methanol (< 5 ppm, detection limit).[150]

Unknown Structural Configuration

Cassia Angustifolia Seed Polysaccharide
The purified seed galactomannan contains mannose:galactose in a ratio of 2.90:1.[137]

Unknown Structural Configuration – Modified

Hydrolyzed Starch

Composition/Properties data on two hydrolyzed starch products are available **(See Table 5)**.[138,139]

Table 5. Composition/Properties Data on Two Hydrolyzed Starch (unknown structural configuration – modified) Products.[138,139]

Product 1	Product 2
G1 (glucose): 2% (not more than 5% for the specification)	G1 (glucose): 2.5% (not more than 5% for the specification)
G2 (maltose): 7%	G2 (maltose): 6%
G3 (maltotriose)*: 10%	G3 (maltotriose)*: 9.5%
G4 (maltotetraose)**: 53% (not less than 50% for the specification)	G4 (maltotetraose)**: 74% (not less than 70% for the specification)
G5 (maltopentaose)***: 2%	G5 (maltopentaose)***: 0.5%
≥ G6****: 26%	≥ G6****: 8%
Loss on drying (water content): ≈ 25% (solids specification: not less than 74%)	Loss on drying (water content): ≈ 28% (solids specification: not less than 72%)
Residue on ignition: ≤ 0.05%	Residue on ignition: ≤ 0.05%
Heavy metals (as lead): ≤ 5 ppm	Heavy metals (as lead): ≤ 5 ppm
Arsenic (as As_2O_3): ≤ 2 ppm	Arsenic (as As_2O_3): ≤ 2 ppm

*_O_-α-glucopyranosyl-(1→4)-_O_-α-D-glucopyranosyl-(1→4)-D-glucose (maltotriose)

**_O_-α-glucopyranosyl-[(1→4)-_O_-α-D-glucopyranosyl]$_2$-(1→4)-D-glucose (maltotetraose)

***_O_-α-glucopyranosyl-[(1→4)-_O_-α-D-glucopyranosyl]$_3$-(1→4)-D-glucose (maltopentaose)

****_O_-α-glucopyranosyl-[(1→4)-_O_-α-D-glucopyranosyl]$_n$-(1→4)-D-glucose (n ≥ 4)

Table 6. Current Frequency and Concentration of Use According to Duration and Type of Exposure.[16,17,18,19]

	Maltodextrin		Glucomannan		Agar	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	542	0.00001-4	NR	0.3-17	67	0.002-1
Duration of Use						
Leave-On	327	0.00001-3	NR	NR	49	0.002-1
Rinse off	188	0.00006-3	NR	0.3-17	17	0.0043-0.015
Diluted for (bath) Use	27	0.22-4	NR	NR	1	NR
Exposure Type						
Eye Area	42	0.001-2.5	NR	17	3	1
Incidental Ingestion	13	0.00075-0.6	NR	NR	NR	NR
Incidental Inhalation- Sprays	189	0.00012-0.38	NR	NR	24	0.0075-1*
Incidental Inhalation- Powders	178	0.005-1	NR	NR	25	0.0075**
Dermal Contact	377	0.00001-4	NR	0.3-17	64	0.002-1
Deodorant (underarm)	NR	0.0045-0.12	NR	NR	NR	NR
Hair - Non-Coloring	80	0.00012-2	NR	NR	3	1
Hair-Coloring	65	0.0001-0.0033	NR	NR	NR	NR
Nail	NR	0.0015-3	NR	NR	NR	NR
Mucous Membrane	80	4	NR	NR	5	NR
Baby Products	2	NR	NR	NR	NR	NR
	Agarose		**Algin**		**Alginic Acid**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	10	0.2-0.7	326	0.001-50	13	NR
Duration of Use						
Leave-On	10	0.2-0.7	194	0.001-18	12	NR
Rinse off	NR	NR	131	0.01-50	1	NR
Diluted for (bath) Use	NR	NR	1	0.1	NR	NR
Exposure Type						
Eye Area	NR	NR	40	0.025-0.75	3	NR
Incidental Ingestion	NR	NR	NR	1.1	NR	NR
Incidental Inhalation- Sprays	1	NR	111	0.001-0.025	6	NR
Incidental Inhalation- Powders	1	NR	119	0.025	6	NR
Dermal Contact	10	0.2-0.7	315	0.001-50	13	NR
Deodorant (underarm)	9	0.7	NR	0.001	NR	NR
Hair - Non-Coloring	NR	NR	3	0.001-0.05	NR	NR
Hair-Coloring	NR	NR	1	1.3	NR	NR
Nail	NR	NR	1	0.002	NR	NR
Mucous Membrane	NR	NR	3	0.01-1.1	NR	NR
Baby Products	NR	NR	4	NR	1	NR
	Amylodextrin		**Astragalus Gummifer Gum**		**Avena Sativa (Oat) Starch**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	2	0.00004	7	NR	5	0.1-9.5
Duration of Use						
Leave-On	2	NR	5	NR	3	0.1-9.5
Rinse off	NR	0.00004	2	NR	2	3.6
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	NR	NR	NR	NR	NR	NR
Incidental Ingestion	NR	NR	NR	NR	NR	NR
Incidental Inhalation- Sprays	1	NR	3	NR	2	0.1-9.5
Incidental Inhalation- Powders	1	NR	3	NR	3	0.1
Dermal Contact	2	NR	4	NR	5	0.1-9.5
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	NR	0.00004	2	NR	NR	NR
Hair-Coloring	NR	NR	1	NR	NR	3.6
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	NR	NR	NR	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR

Table 6. Current Frequency and Concentration of Use According to Duration and Type of Exposure.[16,17,18]

	Calcium Alginate		Carrageenan		Cassia Angustifolia Seed Polysaccharide	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	9	0.01-3	249	0.003-15.7	36	0.002-0.75
Duration of Use						
Leave-On	9	0.01-3	181	0.003-15.7	35	0.002
Rinse off	NR	0.01	63	0.003-3.7	1	0.025-0.75
Diluted for (bath) Use	NR	NR	5	0.1-3	NR	NR
Exposure Type						
Eye Area	NR	NR	18	0.2-3.7	3	NR
Incidental Ingestion	NR	NR	25	1-1.1	3	0.002
Incidental Inhalation- Sprays	2	0.016-1	118	0.03-15.7*	15	0.0025*-0.075*
Incidental Inhalation- Powders	3	0.4-3	11	NR	21	0.0025**-0.025**
Dermal Contact	9	0.01-3	206	0.003-3.7	33	0.0025-0.025
Deodorant (underarm)	NR	0.016-1	NR	NR	NR	NR
Hair - Non-Coloring	NR	NR	14	0.003-15.7	NR	0.025-0.75
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	2	NR	NR	NR
Mucous Membrane	NR	NR	35	0.1-3	3	0.002
Baby Products	NR	NR	1	NR	NR	NR
	Cichorium Intybus (Chicory) Root Oligosaccharides		**Corn Starch Modified**		**Cyclodextrin**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	2	NR	86	0.0062-45.7	128	0.000025-4
Duration of Use						
Leave-On	2	NR	75	0.12-45.7	101	0.000025-4
Rinse off	NR	NR	10	0.0062-3	26	0.0042-1.6
Diluted for (bath) Use	NR	NR	1	9	1	NR
Exposure Type						
Eye Area	NR	NR	7	0.9-8	19	0.05-0.25
Incidental Ingestion	NR	NR	2	0.4	2	0.1
Incidental Inhalation- Sprays	2	NR	48	0.45-45.7*	69	0.08-2.5
Incidental Inhalation- Powders	2	NR	33	0.44**-15	59	0.2
Dermal Contact	2	NR	59	0.0062-15	118	0.0005-4
Deodorant (underarm)	NR	NR	NR	0.12	NR	2.5-4
Hair - Non-Coloring	NR	NR	17	0.45-45.7	5	0.000025-1.6
Hair-Coloring	NR	NR	4	NR	3	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	NR	6	0.0062-9	4	0.1-0.73
Baby Products	NR	NR	2	NR	NR	NR
	Cyclodextrin Laurate		**Dextrin**		**Dextrin Myristate**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	5	0.0035	177	0.000008-43	NR	0.05-19
Duration of Use						
Leave-On	5	0.0035	159	0.000008-30	NR	0.094-19
Rinse off	NR	NR	18	0.001-43	NR	0.05-7
Diluted for (bath) Use	NR	NR	NR	5	NR	NR
Exposure Type						
Eye Area	2	NR	21	0.000008-30	NR	0.094-19
Incidental Ingestion	NR	NR	1	0.008	NR	7-15
Incidental Inhalation- Sprays	3	NR	95	0.00037-2.8	NR	0.099-18
Incidental Inhalation- Powders	3	0.0035**	96	0.0044-2.8	NR	0.3**-16**
Dermal Contact	5	0.0035	168	0.000008-43	NR	0.05-19
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	NR	NR	2	0.00026-0.001	NR	0.099-1
Hair-Coloring	NR	NR	2	NR	NR	NR
Nail	NR	NR	4	0.2	NR	NR
Mucous Membrane	NR	NR	3	0.008-5	NR	7-15
Baby Products	NR	NR	NR	NR	NR	NR

Table 6. Current Frequency and Concentration of Use According to Duration and Type of Exposure.[16,17,18]

	Dextrin Palmitate		**Dextrin Palmitate/Ethylhexanoate**		**Dextrin Palmitate/Stearate**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	77	0.0001-16.8	4	NR	NR	0.1-18
Duration of Use						
Leave-On	71	0.0001-16.8	4	NR	NR	0.1-18
Rinse off	6	0.0002-0.0097	NR	NR	NR	NR
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	13	0.0001-2	NR	NR	NR	0.3-18
Incidental Ingestion	37	0.1-16.8	2	NR	NR	4.5-5
Incidental Inhalation- Sprays	5	NR	1	NR	NR	NR
Incidental Inhalation- Powders	5	0.1-0.5**	1	0.1-3**	NR	0.1-3**
Dermal Contact	33	0.0001-13	2	NR	NR	0.1-10
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	NR	NR	NR	NR	NR	NR
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	0.025	NR	NR	NR	NR
Mucous Membrane	38	0.1-16.8	2	NR	NR	4.5-5
Baby Products	NR	NR	NR	NR	NR	NR
	Galactoarabinan		**Glyceryl Alginate**		**Glyceryl Starch**	
	# of Uses	# of Uses	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	97	NR	NR	0.5	1	4
Duration of Use						
Leave-On	73	NR	NR	0.5	NR	4
Rinse off	24	NR	NR	NR	1	NR
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	21	NR	NR	NR	NR	NR
Incidental Ingestion	2	NR	NR	NR	NR	NR
Incidental Inhalation- Sprays	21	NR	NR	NR	NR	NR
Incidental Inhalation- Powders	21	NR	NR	0.5**	NR	4**
Dermal Contact	76	NR	NR	0.5	1	4
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	9	NR	NR	NR	NR	NR
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	5	NR	NR	NR	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR
	Hydrogenated Starch Hydrolysate		**Hydrolyzed Corn Starch Octenylsuccinate**		**Hydrolyzed Pectin**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	60	0.00007-3.8	13	0.06-0.67	14	NR
Duration of Use						
Leave-On	41	0.00007-0.75	11	0.06	12	NR
Rinse off	19	0.13-3.8	2	0.18-0.67	2	NR
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	1	0.00007-0.5	NR	NR	1	NR
Incidental Ingestion	1	0.065-3.8	NR	NR	NR	NR
Incidental Inhalation- Sprays	33	3.8*	7	NR	10	NR
Incidental Inhalation- Powders	29	0.0007**-0.54**	7	NR	10	NR
Dermal Contact	49	0.00007-0.75	13	0.06-0.67	14	NR
Deodorant (underarm)	NR	NR	3	NR	NR	NR
Hair - Non-Coloring	10	0.13	NR	NR	NR	NR
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	2	0.065-3.8	NR	0.67	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR

Table 6. Current Frequency and Concentration of Use According to Duration and Type of Exposure.[16,17,18]

	Hydrolyzed Starch		Hydrolyzed Wheat Starch		Hydroxyethyl Cyclodextrin	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	NR	0.000013-0.00046	274	0.000003-0.31	NR	1.2
Duration of Use						
Leave-On	NR	0.00046	118	0.00005-0.31	NR	1.2
Rinse off	NR	0.000013	156	0.000003-0.25	NR	NR
Diluted for (bath) Use	NR	NR	4	0.000003	NR	NR
Exposure Type						
Eye Area	NR	NR	6	0.03-0.038	NR	1.2
Incidental Ingestion	NR	NR	NR	NR	NR	NR
Incidental Inhalation- Sprays	NR	0.00046*	66	0.00005-0.02	NR	NR
Incidental Inhalation- Powders	NR	NR	6	0.0002**-0.06**	NR	NR
Dermal Contact	NR	NR	58	0.000003-0.06	NR	1.2
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	NR	0.00046	186	0.000003-0.31	NR	NR
Hair-Coloring	NR	0.000013	26	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	NR	47	0.000003-0.003	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR

	Hydroxypropyl Cyclodextrin		Hydroxypropyltrimonium Hydrolyzed Corn Starch		Hydroxypropyltrimonium Hydrolyzed Wheat Starch	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%
Totals/Conc. Range	53	0.00001-2	11	0.19-0.65	8	NR
Duration of Use						
Leave-On	52	0.00001-2	3	0.24-0.65	NR	NR
Rinse off	1	0.02-0.1	8	0.19-0.43	8	NR
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	13	0.02-1.3	NR	0.65	NR	NR
Incidental Ingestion	NR	0.75	NR	NR	NR	NR
Incidental Inhalation- Sprays	33	0.34-1	3	0.24*	NR	NR
Incidental Inhalation- Powders	29	0.1-2	NR	NR	NR	NR
Dermal Contact	50	0.00001-2	NR	0.65	8	NR
Deodorant (underarm)	1	0.34-2	NR	NR	NR	NR
Hair - Non-Coloring	2	1	11	0.19-0.43	NR	NR
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	0.02	NR	NR	NR	NR
Mucous Membrane	NR	0.75	NR	NR	8	NR
Baby Products	NR	NR	NR	NR	NR	NR

	Hydroxypropyl Starch		Hydroxypropyltrimonium Maltodextrin Crosspolymer		Inulin	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	9	0.25-8.2	NR	0.00045	41	0.0005-3
Duration of Use						
Leave-On	8	0.25-8.2	NR	0.00045	14	0.0005-3
Rinse off	1	0.5-6	NR	NR	27	0.25
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	1	NR	NR	NR	1	0.0005
Incidental Ingestion	NR	NR	NR	NR	NR	NR
Incidental Inhalation- Sprays	6	0.25-0.88	NR	NR	8	NR
Incidental Inhalation- Powders	NR	8.2**	NR	NR	9	0.0008**-2.5**
Dermal Contact	3	0.5-8.2	NR	0.00045	22	0.0005-3
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	6	0.25-1.4	NR	NR	18	NR
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	0.5	NR	NR	4	0.25
Baby Products	NR	NR	NR	NR	1	NR

Table 6. Current Frequency and Concentration of Use According to Duration and Type of Exposure.[16,17,18]

	Laurdimonium Hydroxypropyl Hydrolyzed Wheat Starch		**Mannan**		**Methyl Cyclodextrin**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	6	0.017	19	0.01-0.25	20	4-5
Duration of Use						
Leave-On	NR	NR	16	0.01-0.25	20	4-5
Rinse off	6	0.017	3	NR	NR	NR
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	NR	NR	NR	NR	NR	NR
Incidental Ingestion	NR	NR	NR	NR	NR	NR
Incidental Inhalation- Sprays	NR	NR	11	NR	10	5
Incidental Inhalation- Powders	NR	NR	11	0.01**	NR	NR
Dermal Contact	6	0.017	17	0.01-0.25	19	4-5
Deodorant (underarm)	NR	NR	NR	NR	3	NR
Hair - Non-Coloring	NR	NR	2	NR	1	NR
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	6	0.017	NR	NR	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR
	Pectin		**Polianthes Tuberosa Polysaccharide**		**Potassium Alginate**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	87	0.0001-9	2	0.001-0.1	37	1
Duration of Use						
Leave-On	33	0.001-0.05	2	0.001-1	1	1
Rinse off	54	0.0001-9	NR	NR	36	NR
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	4	NR	NR	NR	NR	NR
Incidental Ingestion	NR	0.09-9	NR	NR	NR	NR
Incidental Inhalation- Sprays	25	0.05	2	0.001-0.1*	1	NR
Incidental Inhalation- Powders	17	NR	2	0.001-0.05**	1	NR
Dermal Contact	57	0.05	2	0.001-0.1	37	1
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	30	0.0001-0.05	NR	NR	NR	NR
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	1	0.09-9	NR	NR	NR	NR
Baby Products	1	NR	NR	NR	NR	NR
	Potato Starch Modified		**Propylene Glycol Alginate**		**Pueraria Lobata Starch**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	61	0.3-1.3	16	0.00001-0.15	NR	3.6
Duration of Use						
Leave-On	40	0.3-1.3	16	0.00001-0.15	NR	NR
Rinse off	21	1.3	NR	NR	NR	3.6
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	NR	NR	2	NR	NR	NR
Incidental Ingestion	NR	NR	NR	NR	NR	NR
Incidental Inhalation- Sprays	9	1.3*	9	0.005-0.03*	NR	NR
Incidental Inhalation- Powders	5	0.3**	9	0.00001**-0.15**	NR	NR
Dermal Contact	11	0.3-1.3	15	0.00001-0.15	NR	NR
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	49	1.3	1	0.005-0.03	NR	NR
Hair-Coloring	1	NR	NR	NR	NR	3.6
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	NR	NR	NR	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR

Table 6. Current Frequency and Concentration of Use According to Duration and Type of Exposure.[16,17,18]

	Sodium Carboxymethyl Starch		**Sodium Carrageenan**		**Sodium Hydrolyzed Potato Starch Dodecenylsuccinate**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	11	0.05-4.7	3	NR	2	NR
Duration of Use						
Leave-On	3	1.9-4.7	1	NR	NR	NR
Rinse off	8	0.05-2.5	2	NR	2	NR
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	1	4.7	NR	NR	NR	NR
Incidental Ingestion	NR	NR	2	NR	NR	NR
Incidental Inhalation- Sprays	NR	NR	1	NR	NR	NR
Incidental Inhalation- Powders	NR	NR	1	NR	NR	NR
Dermal Contact	2	0.05-4.7	1	NR	NR	NR
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	1	1.9	NR	NR	2	NR
Hair-Coloring	8	2.5	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	NR	2	NR	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR
	Sodium Oxidized Starch Acetate/Succinate		**Sodium Starch Octenylsuccinate**		**Solanum Tuberosum (Potato Starch)**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	7	0.05	35	0.0001-0.26	4	3.4-3.6
Duration of Use						
Leave-On	1	0.05	22	0.0001-0.26	2	NR
Rinse off	5	NR	13	0.0023-0.026	2	3.4-3.6
Diluted for (bath) Use	1	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	NR	NR	1	NR	NR	NR
Incidental Ingestion	NR	NR	NR	0.026	NR	NR
Incidental Inhalation- Sprays	1	0.05	16	0.048-0.05	1	NR
Incidental Inhalation- Powders	1	NR	15	NR	1	NR
Dermal Contact	3	NR	21	0.048-0.26	3	NR
Deodorant (underarm)	NR	0.05	4	0.048	NR	NR
Hair - Non-Coloring	4	NR	12	0.0001-0.05	1	3.4
Hair-Coloring	NR	NR	1	NR	NR	3.6
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	2	NR	1	0.026	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR
	Starch Acetate		**Starch Diethylaminoethyl Ether**		**Starch Hydroxypropyltrimonium Chloride**	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	11	2	1	NR	18	0.002-1.2
Duration of Use						
Leave-On	1	NR	NR	NR	1	0.02-1.2
Rinse off	10	2	1	NR	17	0.002-0.39
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	NR	NR	NR	NR	NR	NR
Incidental Ingestion	NR	NR	NR	NR	NR	NR
Incidental Inhalation- Sprays	NR	NR	NR	NR	1	0.05-1.2*
Incidental Inhalation- Powders	NR	NR	NR	NR	NR	0.02**
Dermal Contact	NR	NR	1	NR	2	0.02
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	11	2	NR	NR	16	0.002-1.2
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	NR	NR	NR
Mucous Membrane	NR	NR	1	NR	2	NR
Baby Products	NR	NR	NR	NR	2	NR

Table 6. Current Frequency and Concentration of Use According to Duration and Type of Exposure.[16,17,18]

	Stearoyl Inulin		Sterculia Urens Gum		Tamarindus Indica Seed Gum	
	# of Uses	Conc. (%)	# of Uses	Conc. (%)	# of Uses	Conc. (%)
Totals/Conc. Range	9	0.44-4.8	NR	0.2-0.7	NR	0.01-0.3
Duration of Use						
Leave-On	9	0.44-4.8	NR	0.2-0.7	NR	0.05-0.3
Rinse off	NR	NR	NR	NR	NR	0.01-0.25
Diluted for (bath) Use	NR	NR	NR	NR	NR	NR
Exposure Type						
Eye Area	7	0.44-4.8	NR	NR	NR	NR
Incidental Ingestion	NR	NR	NR	NR	NR	NR
Incidental Inhalation- Sprays	NR	NR	NR	NR	NR	NR
Incidental Inhalation- Powders	NR	NR	NR	NR	NR	0.3**
Dermal Contact	9	0.44-4.8	NR	0.7	NR	0.01-0.3
Deodorant (underarm)	NR	NR	NR	NR	NR	NR
Hair - Non-Coloring	NR	NR	NR	NR	NR	0.25
Hair-Coloring	NR	NR	NR	NR	NR	NR
Nail	NR	NR	NR	0.2	NR	NR
Mucous Membrane	NR	NR	NR	NR	NR	NR
Baby Products	NR	NR	NR	NR	NR	NR
	Tapioca Starch		**Triticum Vulgare (Wheat) Starch**			
	# of Uses	Conc. (%)	# of Uses	Conc. (%)		
Totals/Conc. Range	154	0.45-33	27	0.01-6		
Duration of Use						
Leave-On	124	0.5-33	17	0.01-6		
Rinse off	28	0.45-15	9	0.03-3.6		
Diluted for (bath) Use	2	0.86-32	1	NR		
Exposure Type						
Eye Area	13	NR	5	NR		
Incidental Ingestion	NR	NR	2	0.01		
Incidental Inhalation- Sprays	76	1-15*	1	NR		
Incidental Inhalation- Powders	84	3.7-33	9	NR		
Dermal Contact	115	0.5-33	24	0.03-6		
Deodorant (underarm)	NR	NR	NR	NR		
Hair - Non-Coloring	18	0.45-15	1	NR		
Hair-Coloring	8	3.6	NR	3.6		
Nail	NR	NR	NR	NR		
Mucous Membrane	4	0.86-32	6	0.01		
Baby Products	1	NR	NR	NR		

NR = Not Reported; Totals = Rinse-off + Leave-on + Diluted for (Bath)Use Product Uses.
*It is possible that these products may be sprays, but it is not specified whether the reported uses are sprays.
**It is possible that these products may be powders, but it is not specified whether the reported uses are powders.
***Not specified whether a powder or a spray, so this information is captured for both categories of incidental inhalation
Note: Because each ingredient may be used in cosmetics with multiple exposure types, the sum of all exposure type uses may not equal the sum total uses.

Table 7. Acute Toxicity Studies on Polysaccharide Gums

Inhalation

Branched - Unmodified

Glucomannan (in konjac flour): An acute inhalation toxicity study on glucomannan was performed using male and female rats (number and strain not stated). An LC50 of > 0.0015 mg/l was reported.[151]

Oral

Branched - Unmodified

Glucomannan: Male and female mice (number and strain not stated). LD_{50} > 2,800 mg/kg body weight. No abnormalities with respect to the following: appearance, behavior, body weight changes, occult blood in the urine and feces, or macroscopic findings.[152]

Glucomannan (in konjac flour): Male and female rats (number and strain not stated. LD_{50} > 5,000 mg/kg body weight.[151]

Sterculia Urens Gum: Vehicle: corn oil. 5 fasted male Sprague-Dawley rats. LD_{50} > 10,000 mg/kg body weight. Transient depression, but no other toxic effects.[153]

Branched - Modified

Calcium Starch Isododecenylsuccinate: Material structurally similar to this gum tested. 5 male and 5 female Wistar albino rats. OECD Guideline 401 test protocol. Dosing followed by 14-day observation period. No abnormal systemic signs. LD_{50} > 5,000 mg/kg body weight.[63,64,154]

Corn Starch Modified: Vehicle: distilled water. 5 male and 5 female Wistar albino rats. Organisation for Economic Co-operation and Development (OECD) 401 protocol. 14-day observation period. Alopecia in one animal. LD_{50} > 2,000 mg/kg body weight.[66]

Dextrin Myristate: Rats (number and strain not stated). LD_{50} > 2,000 mg/kg body weight.[67]

Dextrin Palmitate: Rats (number and strain not stated). LD_{50} > 2,000 mg/kg body weight.[68,69]

Potato Starch Modified: 30% aqueous solution. Albino rats (5 males, 5 females). OECD 401 protocol. 14-day observation period. Soft stool (1 female); and no other signs. Body weight changes at necropsy normal. LD_{50} > 5,000 mg/kg body weight.[70,155]

Stearoyl Inulin: Rats (number and strain not stated). Protocol not stated. LD_{50} > 2,000 mg/kg body weight.[71,72]

Dermal

Branched - Unmodified

Glucomannan (in konjac flour): Male and female rabbits (number and strain not stated). Protocol not stated. LD_{50} > 2,000 mg/kg body weight.[151]

Branched - Modified

Carboxymethyl Inulin: 31.1% aqueous carboxymethyl inulin. 10 adult Dunkin–Hartley albino guinea pigs (4 weeks old). Maximization test. No mortality occurred and no clinical signs of systemic toxicity. Body weights and weight gains similar in treated and control groups.[156]

Corn Starch Modified: Corn starch modified (Amaze® [28-1890]) in distilled water (30% solids). 5 male and 5 female New Zealand White rabbits. OECD 402 protocol. 14-day observation period. Nine of 10 rabbits survived. LD_{50} > 2,000 mg/kg body weight.[66]

Dextrin Myristate: Rats (number and strain not stated). Occlusive dressing technique (details not included). LD_{50} > 2,000 mg/kg body weight.[67]

Dextrin Palmitate: Rats (number and strain not stated). Occlusive dressing technique (details not included). LD_{50} > 2,000 mg/kg body weight.[68,69]

Potato Starch Modified: 10 rats (strain not specified). OECD 402 test guideline. LD_{50} > 2,000 mg/kg body weight.[155]

Potato Starch Modified: 18.5% solids aqueous solution. 10 New Zealand White rabbits (5 males and 5 females). Semi-occlusive patch application. Dose per cm^2 was not stated. Very slight to slight erythema/edema at application sites (all animals); reactions had cleared by 72 h. Signs of local irritation may have been due to mechanical trauma. LD_{50} > 2,000 mg/kg body weight.[70]

Intravenous

Linear Polysaccharides and Their Salts

Carrageenan and Potassium Carrageenan: ι-carrageenan (one subtype of carrageenan with a specific number and position of sulfate groups on the repeating galactose units) or potassium carrageenan (2 mg in phosphate-buffered saline [PBS]). Groups of 5 female MF1 mice. i.v. injection (lateral tail vein). Controls injected with PBS (0.3 ml). Animals killed at 1 h and 24 h post-injection, and tissues prepared for microscopic examination. Carrageenan persisted for at least 6 months in livers and kidneys. Within 24 h of i.v. injection, damage to liver Küpffer cells and changes in the microcirculation characteristic of disseminated intravascular coagulation (DIC) in the liver and kidney observed. No adverse effects in hepatocytes, but chronic renal damage observed. ι-carrageenan less toxic to liver and kidney, compared to the potassium carrageenan (less pure, compared to ι-carrageenan).[157]

Carrageenan and Potassium Carrageenan: ι-carrageenan or potassium carrageenan in saline (0.5 ml or 1 ml i.v. injection). Groups of 9 to 15 female CAF_1 mice (Balb/c x A/He). 7- or 14-day observation period. Treatment with either compound induced anemia, granulocytosis, and early profound thrombocytopenia. Treatment with ι-carrageenan caused an early lymphocytosis, and both compounds induced lymphopenia by 18 h post-treatment. Treatment with either compound was associated with an early moderate reduction in the number of nucleated cells and granulocyte/macrophage colony-forming cells per femur. Each compound induced splenomegaly, and ι-carrageenan-treated mice developed hypoplasia of the thymus by 18 h post-injection. Sustained increase in numbers of colony-forming cells in spleen after treatment with each compound.[158]

Table 7. Acute Toxicity Studies on Polysaccharide Gums

Intrapleural
Linear Polysaccharides and Their Salts
Carrageenan: Groups of 6 adult female Balb/c mice (6 to 7 weeks old). One group received single intrapleural injection of 0.1 ml sterile saline (0.9% NaCl) and λ-carrageenan (one subtype of carrageenan with a specific number and position of sulfate groups on the repeating galactose units; 1% in solvent [not stated]), which induced pleurisy. Another group each received single intrapleural injection of 1% λ-carrageenan (0.1 ml) only. Animals were killed, and lung tissue samples obtained for microscopic examination at 4 h and 24 h post-injection. Dense inflammation with lobar lung pneumonia and thickened alveolar septum (with occasionally obliterated alveoli) were observed.[159]
Carrageenan: Injection of 2% λ-carrageenan in saline (200 mg/kg) into pleural cavity. Groups of 10 mice. Dosing caused pleurisy, characterized by marked accumulation of fluid and the migration of leukocytes to the site of inflammation in lung.[160]
Transbronchial
Linear Polysaccharides and Their Salts
Carrageenan: Transbronchial injection of 0.75% carrageenan in physiological saline. 27 male albino rabbits. Surviving animals were killed according to the following schedule: 2 at 24 h; 3 each at 3 days, 1 and 2 weeks, and 1 month; 5 at 2 months; and 8 at 4 months. Pneumonia, followed by emphysema in the insulted lung, observed. Of the 8 animals injected with carrageenan and killed at 4 months, 3 were deemed inappropriate for morphometry because of developing fibrosis, abscesses and/or emphysematous bullae in the lungs. Thus, the lungs (mild to severe erythema observed) of the remaining 5 animals injected with carrageenan and of the 5 control rabbits killed at 4 months were prepared for morphometric analysis. Scattered infiltration of polymorphonuclear leukocytes throughout the affected lobe, subsequently replaced by accumulation of carrageenan-laden macrophages; changes lasted for 1 to 2 months. Enlargement of alveoli and alveolar ducts observed at 2 weeks to 2 months post-injection, and pulmonary emphysema observed at 4 months. The lobes not injected with carrageenan had normal appearance throughout study.[161]

Table 8. Repeated Dose Toxicity Studies on Polysaccharide Gums

Oral - Non-Human

Linear Polysaccharides and Their Salts

Algin: 25% Sodium alginate (also known as algin) in diet. Mice (75 males and 75 females). Feeding with sodium alginate in the diet for 89 weeks. At week 87, half of the surviving male and female mice in each test group placed on control diet (containing 55% pregelatinized potato starch). During feeding period, dietary levels of test substances gradually increased until diets contained (by weight) 25% sodium alginate. All survivors killed during weeks 89 to 92. Sodium alginate caused increased water consumption, distinct caecal and colonic enlargement, and a slightly increased incidence of intratubular nephrosis. Sodium alginate was nephrotoxic, causing increased kidney weights, distension of the renal calyx and high incidence of dilated distal tubules.[162]

Carrageenan: 25,000 ppm or 50,000 ppm kappa carrageenan. Groups of Fischer 344 rats (20/sex/group). Feeding in diet for 90 days. Clinical signs limited to soft feces in high dose rats, and to a lesser extent, in low dose rats. No treatment-related effects on body weights, urinalysis, hematology or clinical chemistry parameters, or on organ weights or ophthalmic, macroscopic or microscopic findings. Gastrointestinal tract appeared normal in detailed histopathological evaluation. NOAEL = 50,000 ppm (mean calculated test material consumption of 3394 ± 706 mg/kg/day in males and 3867 ± 647 mg/kg/day in females).[113]

Carrageenan: kappa/lambda-carrageenan (from *C. crispus* or *G. mamillosa)* at concentrations of 0, 0.1, 5, 15, or 25%. Five male and five female mice of 2 unidentified strains. Lifetime dietary feeding had no adverse effect. Same test material and dietary concentrations. Five male and 5 female rats of 2 unidentified strains. Lifetime dietary feeding. Evidence of hepatic cirrhosis, only at the 25% concentration, with no effect on mortality.[62]

Carrageenan: Extracts of kappa-carrageenan (from *Hypnea musciformis* or *Irideae crispata)* at concentration of 1% or 5%. Groups of 15 male and female Sprague-Dawley rats. Feeding in diet for 1 year. Weight loss ($p = 0.05$) in all treatment groups, compared to control (alphacel) group. Livers of rats fed 1% concentration normal at gross and microscopic examination. Livers from rats given 5% kappa-carrageenan from *H. musciformis* normal at gross and microscopic examination, except for nodules in 2 of 12 livers. Gross examination of livers from rats fed 5% kappa-carrageenan (from *I. crispata*) showed decreased size, rough surface, and vascularization in 10/13 rats, probably treatment-related. Microscopically, these livers were normal, except for focal necrosis in 1 of 10 livers. No evidence of storage of carrageenan-like material (metachromatic) in liver cells of any of the treated rats, and no fibrillar material observed using electron microscopy. No changes observed in stools of rats receiving 1% of either carrageenan. Loose stools in female rats given 5% kappa-carrageenan from *I. crispata* and in males given either carrageenan at 5% concentrations. Blood found sporadically in stools, but frequency was not significant.[62]

Carrageenan: kappa/lambda-carrageenan. Groups of 19 male and 21 female rhesus monkeys. Feeding (gavage) with 0, 50, 200, or 500 mg/kg body weight (6 days/week for five years, and dietary feeding for an additional 2.5 years. Random distribution of loose stools, chronic intestinal disorders, poor appetite, and emaciation. Stool consistency decreased in dose-related trend over entire 7.5 years of the study; findings of fecal occult blood increased in similar fashion. Mean survival time similar in all groups; no gross or microscopic changes in tissues examined. Sporadic differences in body weight observed randomly. Females had significant body-weight depression (not dose-related) in last 2.5 years of study. No consistent, statistically significant changes in hematological or clinical chemical values, absolute organ weights, or organ-to-body weight ratios after 7.5 years of feeding. Cytochemical and ultrastructural observations revealed no storage of carrageenan-like material in livers, obtained at biopsy or in other organs obtained at necropsy; no dose-related gross or microscopic changes in other tissues.[62]

Inulin: 7.5% inulin. 20 Wistar rats of the Crl:(WI)BR strain (10 males, 10 females). Daily dietary feeding for 13 weeks. No remarkable microscopic or macroscopic findings.[163]

Branched - Unmodified

Arabinoxylan: Wheat bran extract (~ 80% arabinoxylan oligopeptides) at concentrations of 0.3%, 1.5%, and 7.5%. 3 groups of 20 Wistar rats of the Crl:(WI)BR strain (10 males/group, 10 females/group). Feeding resulted in average daily intakes of 0.2 g/kg (0.3% concentration), 0.9 g/kg (1.5%), and 4.4 g/kg (7.5%) for 13 weeks. No evidence of test substance-related adverse macroscopic or microscopic findings. At histopathological examination, minimal bilateral hypertrophy of renal cortical tubules in males and females, particularly in highest-dose group. Findings were not accompanied by degenerative changes or changes in kidney weight, and were considered non-toxic and suggestive of an adaptive response. No remarkable findings in control rats fed basal diet. NOAEL = 4.4 g/kg/day.[163]

Ghatti Gum: Ghatti gum concentrations of 0, 0.5, 1.5 and 5%. Groups of Sprague-Dawley rats (10 males/group, 10 females/group). Dietary feeding (in basal diet) for at least 90 days. Ghatti gum intake at 5% dietary level ranged from 3044 to 3825 mg/kg body weight/day. Feed consumption among treated and control groups was similar for males and females. 2 of 10 females in 5% ghatti gum group had a single colon ulcer, with associated acute inflammation. Ulcers were considered sporadic occurrences, possibly attributable to basal diet. NOAEL = 5% in diet; NOAELs for males and females estimated at 3044 and 3309 mg/kg/day, respectively.[164]

Ghatti Gum: 5% Ghatti gum. Groups of 20 female Sprague-Dawley rats. Dietary feeding for at least 90 days. Single colon ulcer, with associated acute inflammation, in 1 of 20 control females given basal diet. Colon ulcer considered sporadic, possibly attributable to basal diet. Statistically significant alterations in clinical chemistry were considered sporadic and unrelated to treatment. Feed consumption among treated and control groups similar for each sex. NOAEL = 5% in diet; NOAELs at 3670 and 3825 mg/kg/day for different control diets.[164]

Glucomannan: 10% konjac (plant consisting mostly of glucomannan). Groups of four male Sprague-Dawley rats were fed either 5% cellulose (control), 10% pectin, or 10% konjac for 28 days. After dosing period, rats were fasted for 24 h, fed 5 g/kg body weight brown rice, and killed 5 h later. No indication of toxicity.[165,166]

Table 8. Repeated Dose Toxicity Studies on Polysaccharide Gums

Branched - Unmodified

Glucomannan: 2.5%, 5%, or 10% refined konjac meal. Groups of 12 five-week-old Sprague-Dawley rats of each sex . Feeding with either a normal basal diet, a hypercholesterolaemic diet (control diet containing 1% cholesterol), or one of three test diets. Because refined konjac meal contains ~ 80% glucomannan, the highest concentration of glucomannan tested was ~ 8%. Four animals of each sex from each group killed after 4, 8, and 12 weeks of feeding. Histological and gross examination of livers from rats fed 1% cholesterol showed spreading fatty degeneration with focal necrosis and a nonspecific inflammation reaction. Similar changes observed in group receiving refined konjac meal at the end of 4 weeks, but the changes disappeared gradually with longer feeding times, and the morphology of the liver was similar to that in the normal control group at the end of 12 weeks. Changes were also observed at gross examination of the liver.[167]

Glucomannan: Basal diet in which 1% of the cornstarch replaced with refined glucomannan (i.e., 1% konjac meal). Groups of 15 Sprague-Dawley rats of each sex. Dietary feeding for 18 months. At the end of feeding period, the animals were killed and the brain, liver, aorta, kidney, spleen, and heart removed. At microscopic examination, the livers of treated rats contained smaller, more lightly stained nuclei and reduced bile-duct proliferation in the portal area. Endothelial cells in the aorta of treated animals were smaller and there was less thickening of the aortic wall. These changes were related to less senescence in the treated group than in the control group. No evidence of treatment-related pathological changes. NOAEL = 1% konjac meal, equivalent to an intake of 500 mg/kg body weight per day.[165]

Pectin and Solanum Tuberosum (Potato) Starch: Test diets containing 5% or 10% pectin-derived acidic oligosaccharides (pAOS). Two groups of F_1 rats (from outbred strain of Wistar rats (Crl:WI(WU); number not stated). Dietary feeding with test (±7 g/kg body weight/day) and control diets for 13 weeks. To keep the total level of added test substance equal in each diet, the low-dose diet (5% pAOS) was adjusted with 5% potato starch. One control group received the standard rodent diet supplemented with 10% potato starch, and the other control group received 10% short-chain FOS (scFOS) in the diet. No treatment- related clinical signs observed, and none of the rats died . Ophthalmoscopic examination did not reveal any treatment-related ocular changes. Neurobehavioral examination and motor activity assessment did not indicate any neurotoxic potential. No relevant differences in body weight, growth rate and feed intake. Macroscopic examination at necropsy did not reveal any adverse effects. Microscopic examination revealed treatment-related histopathological changes in the urinary bladder of animals of the 10% pAOS group. One male and one female of the 5% pAOS group and one male of the control group showed diffuse hyperplasia (very slight). In addition, two males and two females of the 5% pAOS group showed simple hyperplasia in a part of the urinary bladder lining ('focal hyperplasia'). No treatment-related hyperplasia of the transitional epithelium was observed in the kidney. Administration of pAOS at dietary levels up to 10% (equivalent to 7.1 g/kg body weight/day) did not reveal any relevant effects that could be attributed to the ingestion of acidic oligosaccharides.[168]

Starch Acetate: 55% Starch acetate (a chemically modified potato starch) in diet. Mice (75 males and 75 females per test substance). Feeding with starch acetate in the diet for 89 weeks. At week 87, half of the surviving male and female mice placed on control diet (containing 55% pregelatinized potato starch). During feeding period, dietary level of test substance gradually increased until diet contained (by weight) 55% starch acetate. All survivors killed during weeks 89 to 92. Starch acetate caused increased water consumption, distinct caecal and colonic enlargement, and a slightly increased incidence of intratubular nephrosis. Increased incidence of gastric trichobezoars. Concretions in renal pelvis with slight urinary changes, such as increased amounts of amorphous material in the urine and increased urinary calcium content, in the mice fed starch acetate not toxicologically significant. The incidence of intratubular calcinosis or concretions in the pelvic space was not reduced during the recovery period. Caecal and colonic enlargement and changes in urinalysis results were found to be reversible.[162]

Sterculia Urens Gum: 5 non-fasted male Sprague-Dawley rats. Animals intubated with 5 g/kg/day, daily for 5 days. No adverse effects.[153]

Sterculia Urens Gum: 7% (w/w) sterculia urens gum. Albino Wistar rats (rats housed 3 per cage; number tested not stated) Transmission electron microscopy used to study ultrastructure of jejunum, ileum, and cecum after dietary supplementation for 45 days [15 micrographs analyzed] for 45 days. No abnormalities in any of the organelles.[169]

Branched - Modified

Carboxymethyl Inulin: Carboxymethyl inulin (31.1% aqueous). Groups of five male and five female Wistar Crl rats. Doses of 0, 50, 150 and 1000 mg/kg/day (by gavage) for 4 weeks. In all dose groups, no treatment-related effects with respect to: body weight, feed consumption, mortality, hematology, clinical blood chemistry, organ weights or gross or microscopic pathology.[156]

Cyclic

Cyclodextrin: β-cyclodextrin (12,500, 25,000 and 50,000 ppm). Groups of 40 (20 males, 20 females/group) CrI:CD (SD) BR Sprague-Dawley rats. Feeding in the diet for 52 weeks. Control group fed basal diet. The liver and kidney were identified at histopathological examination as target organs for toxicity at concentrations of 50,000 ppm and 25,000 ppm, with the hepatic changes associated with increased plasma liver enzyme and decreased plasma triglyceride concentrations. The only finding for kidneys was a statistically significant ($p < 0.01$) increased incidence of minimal/trace amounts of pigment in the epithelium of the cortical tubules in female rats that received 25,000 ppm or 50,000 ppm β-cyclodextrin in the diet. The "non-toxic dietary inclusion level" of β-cyclodextrin was 12,500 ppm (equivalent to 654 or 864 mg/kg/day for males or females, respectively).[44]

Cyclodextrin: β-cyclodextrin (6200, 12,500 and 50,000 ppm). Groups of 8 (4males, 4 females/group) pure-bred Beagle dogs. Preceding test protocol in rat study used. No pathological evidence of systemic toxicity, although there were minor changes in urinalysis and biochemical parameters and a slightly higher incidence of liquid feces. These changes were considered to be of no toxicological importance. The "non-toxic dietary inclusion level" of β-cyclodextrin was 50,000 ppm (equivalent to 1,831 or 1,967 mg/kg/day for males or females, respectively).[44]

Cycodextrin: γ-cyclodextrin (5%, 10%, or 20%). Groups of 8 (4 males, 4 females) Beagle dogs. Feeding in the diet for 13 weeks. Control group fed basal diet. No treatment-related changes in behavior or appearance and no mortalities. No treatment-related differences with respect to ophthalmoscopic examinations, hematological parameters, clinochemical analyses of the plasma, and semiquantitative urine analyses. Relative ovary weights ignificantly increased in the 10% and 20% concentration groups, but this observation was probably a result of an unusually low ovarian weight in the controls. An increase in relative liver weights in males of the 10% and 20% concentration groups was also considered to lack toxicological relevance, because this observation was not associated with changes in plasma enzyme levels or with histopathological changes. No treatment-related abnormalitiesobserved at necropsy. At microscopic examination, no treatment-related effects in any of the various organs and tissues. Daily consumption of up to 20% γ-cyclodextrin in the diet (≈ 7.7 g/kg body weight in males and 8.3 g/kg body weight in females) did not cause toxicity.[170]

Table 8. Repeated Dose Toxicity Studies on Polysaccharide Gums

Oral - Human

Branched - Unmodified

Sterculia Urens Gum: 5 male volunteers (30 to 56 years old). Ingestion of sterculia urens gum (10.5 g in diet) daily for 21 days. No toxicity or significant effects on plasma biochemistry, hematological indices, or urinalysis parameters were noted.[171]

Branched – Modified

Propylene Glycol Alginate: 5 male volunteers. Following a 7-day control period, the men consumed an amount of propylene glycol alginate equal to 175 mg/kg body weight during the first 7 days of the test period. The amount consumed was increased to 200 mg/kg body weight for the remainder (i.e., 16 days) of the 23 days of dietary supplementation. No significant effect (statistical analysis not performed) on the following: hematological indices, plasma biochemistry parameters, urinalysis parameters, blood glucose levels, plasma insulin concentrations, and expired hydrogen concentrations. Ingestion of propylene glycol alginate caused no adverse dietary or physiological effects. The enzymatic indicators of toxicological effects remained unchanged.[53]

Dermal - Non-Human

Branched - Modified

Carboxymethyl Inulin: 31.1% aqueous carboxymethyl inulin. 10 adult Dunkin–Hartley albino guinea pigs. Maximization test. 5 female guinea pigs (vehicle controls). No mortalities or clinical signs of systemic toxicity were observed. Body weights and weight gains were considered similar when treated and control groups were compared.[156]

Potato Starch Modified: Rats (10 males, 10 females). Applied to skin under occlusive dressing for 28 days (2 g/kg body weight/day) according to OECD 410 test guideline. Sporadic gains and losses of body weight. Compared to the vehicle control group, statistically significant (p value not stated) decrease in body weight gain in treated females during weeks 1 and 4. Clinical biochemical test results indicated statistically significant (p value not stated) decrease in serum triglycerides and slight increase in serum calcium, sodium, and phosphorus in treated males, but not in females. However, none of the other test parameters supported these findings. Decreased organ weights and differences in hematologic test parameters, but these findings were within historical control ranges for this strain of rat. Signs of systemic toxicity not observed at gross examination of treated animals. NOAEL $\geq$ 2,000 mg/kg body weight/day.[155]

Potato Starch Modified: 10% solids aqueous solution. New Zealand albino rabbits (10 males and 10 females) tested; 20 rabbits (controls). Applied to skin under a non-occlusive patch (dose = 2 g/kg bodyweight). Area of application and concentration/dose per cm^2 were not stated. Distilled water, under a non-occlusive patch, applied to controls. Daily evaluations for signs of systemic toxicity, mortality, or morbidity occurred daily; necropsy on day 28. The following considered within normal parameters: body weights, food consumption, gross pathology, and histopathology. Minor differences in organ weight and clinical chemistry changes observed, but considered irrelevant. No significant toxic effects in rabbits.[70]

Table 9. Reproductive and Developmental Toxicity Studies on Polysaccharide Gums

Ingredient	Animals	Procedure	Results
Linear polysaccharides and their salts			
Ammonium Alginate	Fertile eggs from Single-comb White Leghorn chickens	Single injection of ammonium alginate (in corn oil, ≤ 100 µl) into groups of 20 or more eggs; doses up to 0.5 mg/egg)	Injection did not result in significant numbers of abnormal birds.[172]
kappa/lambda-Carrageenan (from *C. crispus*) sodium or calcium salt	Groups of 22 to 27 pregnant CD-1 mice	Oral doses of 10, 45, 470, or 900 mg/kg body weight/day on days 6-15 of gestation	Number of fetal resorptions and/or fetal deaths increased. Dose-dependent decrease in number of live pups and pup weight. Skeletal maturation was retarded. A no-observed-effect level was not reported.[62]
kappa/lambda-Carrageenan (from *C. crispus*) sodium or calcium salt	Groups of 21 to 27 pregnant rats (strain not stated)	Oral doses of 40, 100, 240, or 600 mg/kg body weight/day on days 6-15 of gestation	Increased fetal resorptions, with no decrease in the number of live pups. Dose-dependent increase in incidence of missing skeletal sternebrae.[62]
kappa/lambda-Carrageenan (from *C. crispus*) sodium or calcium salt	Groups of 21 to 24 pregnant rats (strain not stated)	Feeding with 1% or 5% in diet on days 6-16 of gestation	Neither salt was teratogenic.[62]
kappa/lambda-Carrageenan (from *C. crispus*) calcium salt	40 male and 40 female Osborne-Mendel rats	Three-generation study. Feeding with 0.5, 1, 2.5, or 5% in diet 12 weeks prior to mating	In F_{2c} and F_{3c} litters, no specific external, skeletal, or soft-tissue anomaly could be correlated with dosage.[62]
Calcium Carrageenan	Sprague-Dawley rats (number not stated)	Feeding with 0.45, 0.9, or 1.8% in diet prior to mating, during breeding, and throughout gestation, lactation, and post-weaning	No differences between test and negative control groups regarding length of gestation, litter size, or sex distribution.[62,173]
kappa/lambda-Carrageenan (from *C. crispus*) sodium or calcium salt	Groups of 23 to 30 pregnant hamsters (strain not stated)	Oral doses of 40, 100, 240, or 600 mg/kg body weight on days 6-10 of gestation	No significant effect on nidation or on maternal or fetal survival. Some evidence of dose-dependent delay in skeletal maturation.[62]
kappa/lambda-Carrageenan (from *C. crispus*) sodium or calcium salt	Groups of 21 to 26 pregnant hamsters	Feeding with 1% or 5% in diet on days 6-11of gestation	Neither salt was teratogenic.[62]
Carrageenan (sodium or calcium salt) or degraded Carrageenan	21 pregnant female Syrian hamsters per dose of carrageenan; 8 pregnant females per dose of degraded carrageenan	Oral doses of 10, 40, 100, or 200 mg/kg body weight on days 6-10 of gestation	No dose-related teratogenic or fetotoxic effects.[62]
kappa/lambda-Carrageenan (from *C. crispus*) sodium or calcium salt	Groups of 12 to 13 pregnant female rabbits (strain not stated)	Oral doses of 40, 100, 240, or 600 mg/kg body weight on days 6-18 of gestation	The numbers of skeletal or soft tissue abnormalities did not differ from those of controls.[62]

Table 9. Reproductive and Developmental Toxicity Studies on Polysaccharide Gums

Ingredient	Animals	Procedure	Results
Branched - unmodified			
Glucomannan (from *Amorphophallus oncophyllus*)	6 pregnant British short-hair domestic cats	Concentration of 2% in the diet during gestation. Actual intake during week prior to parturition ranged from 0.98 to 3.08 mg/kg body weight per day	All pregnant females completed lactation and a normal gestation period. No adverse effect on mean birth weight or mean litter size.[105]
Pectin-derived acidic oligosaccharides (pAOS)	Groups of 24 (16 females, 8 males per group) parental (F_0) Wistar rats of the crl:WI(WU) outbred strain	Concentrations of 5% or 10% in the diet prior to mating, and throughout mating, gestation, and lactation periods	No effect on estral cycle length and normality. No relevant changes in sperm motility, sperm count, or morphologic changes. No effects on reproductive indices, including litter size, pup viability, and difference in sex ratio.[168]
Sterculia Urens Gum (suspension in anhydrous corn oil)	Groups of 87 to 90 pregnant female Dutch-belted rabbits	Oral doses up to 635 mg/kg/day for 13 consecutive days (gestation daysa 8-18).	Not teratogenic.[174]
Sterculia Urens Gum (suspension in anhydrous corn oil)	Groups of 87 to 90 pregnant female albino CD-1 mice	Oral doses up to 170 mg/kg body weight on days 6 through 15 of gestation	No clearly discernible effect on nidation or on maternal or fetal survival. No difference in soft or skeletal tissue abnormalities between test animals and sham-treated controls. Not teratogenic.[174]
Sterculia Urens Gum (suspension in anhydrous corn oil)	28 pregnant female albino CD-1 mice	Oral dose of 800 mg/kg body weight on days 6 through 15 of gestation	Significant number of maternal deaths (9 of 28). Surving dams were completely normal and delivered normal fetuses, with no effect on rate of nidation, or live pup survival *in utero*. Not teratogenic.[174]
Sterculia Urens Gum (suspension in anhydrous corn oil)	Groups of 87 to 89 pregnant female Wistar-derived albino rats	Oral doses up to 900 mg/kg body weight on days 6 through 15 of gestation	Dams were completely normal and delivered normal fetuses, with no effect on rate of nidation, or live pup survival *in utero*. Not teratogenic.[174]
Branched - modified (i.e., added sidechains are larger than acetate)			
Propylene Glycol Alginate	Fertile eggs from Single-comb White Leghorn chickens	Single injection of propylene glycol alginate (in water, ≤ 100 µl) into groups of 20 or more eggs; doses up to 1 mg/egg)	Injection did not result in significant numbers of abnormal birds.[172]
Cyclic			
γ-Cyclodextrin	Groups of 25 pregnant female Wistar Crl (WI)WU BR rats	Concentrations of 1.5%, 5%, 10%, and 20% in the diet on gestation days 0 to 21.	No fetotoxic embryotoxic, or teratogenic effects. NOAEC ≈ 20% in diet (≈ 11 g/kg body weight per day).[175]
α-Cyclodextrin	Groups of 25 pregnant female Wistar Crl (WI)WU BR rats	Concentrations of 1.5%, 5%, 10%, and 20% in the diet on gestation days 0 to 21.	No fetotoxic embryotoxic, or teratogenic effects. NOAEC = 20% in diet (≈ 13 g/kg body weight per day).[176]
γ-Cyclodextrin	Groups of 16 pregnant female New Zealand White rabbits	Concentrations of 5%, 10%, or 20% in the diet on gestation days 0 to 29.	No effect on reproductive performance, and not fetotoxic, embryotoxic, or teratogenic.[177]
α-Cyclodextrin	Groups of 16 pregnant female New Zealand White rabbits	Concentrations of 5%, 10%, or 20% in the diet on gestation days 0 to 29.	No effect on reproductive performance, and not fetotoxic, embryotoxic, or teratogenic.[178]

Table 10. Genotoxicity of Polysaccharide Gums

Ingredient/Similar Chemical	Strain/cell type	Assay	Dose	Results
		Bacterial Assays		
Linear polysaccharides and their salts				
Carrageenan (natural grade [PNG]) or refined Carrageenan	*Salmonella typhimurium* strain TA100	Ames test	Concentrations up to 100 mg/ml (PNG) and up to 25 mg/ml (refined) without metabolic activation	Not genotoxic.[179]
kappa/lambda-Carrageenan (from *C. crispus*)	*Salmonella typhimurium* strains TA1535, TA1537, and TA1538. *Saccharomyces cerevisiae* strain D4.	Ames test	Test concentrations not stated	Not genotoxic.[62]
PNG or Refined Carrageenan	Mice (strain not stated). *Salmonella typhimurium* strain His G 46	Host-mediated assay	Mice received PNG at oral doses up to 2,500 mg/kg body weight or refined carrageenan at a dose of 700 mg/kg body weight. Bacterial strain tested without metabolic activation	Mutation frequency in injected indicator organism not affected by dosing with carrageenan. Neither PNG nor refined carrageenan was genotoxic.[179]
PNG or Refined Carrageenan	*Bacillus subtilis*	Rec assay for DNA -damaging potential	PNG and refined carrageenan tested at concentrations up to 100 mg/ml and 28 mg/ml, respectively	Neither PNG nor refined carrageenan was genotoxic.[179]
Linear - modified				
Hydrolyzed furcellaran trade name mixture (0.6% hydrolyzed furcellaran, 0.05% concentrate of sea water, 1% phenoxyethanol, and 98.35% water)	*Salmonella typhimurium* strains TA97a, TA98, TA100, and TA 1535; *E. coli* strain WP2uvrA pKM101	Ames test	Doses and presence/absence of activation not stated	Not genotoxic.[73]
Branched - unmodified				
Arabinoxylan	*Salmonella typhimurium* strains TA98, TA 100, TA 1535, and TA 1537; *Escherichia coli* (*E. coli*) strain WP2*uvr*A	Ames test	up to 5,000 µg/plate, with and without metabolic activation	Not genotoxic.[163]
Ghatti gum	*Salmonella typhimurium* strains TA97a, TA98, TA100, and TA 1535; *E. coli* strain WP2uvrA pKM101	Ames test	6 mg/plate, with and without metabolic activation	Not genotoxic.[180]
Glucomannan (in konjac flour)	*Salmonella typhimurium* (5 strains, not stated)	Ames test	With and without metabolic activation (doses not stated)	Not genotoxic.[151]
Pectin-derived acidic oligosaccharides (mixture of linear oligomers and small polymers of galacturonic acid) (for genotoxicity evaluation of Pectin)	*Salmonella typhimurium* strains TA98, TA 100, TA 1535, and TA 1537; *E. coli* strain WP2*uvr*A	Ames test	up to 5,000 µg/plate, with and without metabolic activation	Not genotoxic.[168]

Table 10. Genotoxicity of Polysaccharide Gums

Ingredient/Similar Chemical	Strain/cell type	Assay	Dose	Results
Sterculia urens gum	Mice (strain not stated). *Salmonella typhimurium* strains G46 and TA1530 and *Saccharomyces cerevisiae* strain D3	Host-mediated assay	3 groups of mice intubated with 5,000 mg/kg, 2500 mg/kg, and 30 mg/kg, respectively, followed by injection with tester strains	Not genotoxic in plated tester strains.[153]
Branched - modified (i.e., added sidechains are larger than acetate)				
Carboxymethyl inulin	*Salmonella typhimurium* strains TA98, TA 100, TA 1535, and TA 1537; *Escherichia coli* (*E. coli*) strain WP2*uvr*A	Ames test	Same as above	Not genotoxic.[156]
Calcium Starch Isododecenylsuccinate	*Salmonella typhimurium* strains TA98, TA 100, TA 1535, and TA 1537; *E. coli* strain WP2*uvr*A	Ames test	up to 5,000 µg/plate, with and without metabolic activation	Not genotoxic.[63]
Corn starch modified (Amaze® [28-1890])	*Salmonella typhimurium* strains TA98, TA 100, TA 1535, or TA 1537; *E. coli* strain WP2*uvr*A	Ames test	up to 5,000 µg/plate, with and without metabolic activation	Not genotoxic.[66]
Dextrin myristate (Rheopearl MKL2)	*Salmonella typhimurium* (strains not stated)	Ames test	Doses and presence/absence of activation not stated	Not genotoxic.[67]
Dextrin palmitate (Rheopearl KL2 and Rheopearl TL2)	*Salmonella typhimurium* (strains not stated)	Ames test	Doses and presence/absence of activation not stated	Not genotoxic.[68,69]
Dextrin isostearate (Unifilma HVY)	*Salmonella typhimurium* and *E. coli* (strains not stated)	Ames test	Doses and presence/absence of activation not stated	Not genotoxic.[127]
Sodium Hydrolyzed Potato Starch Dodecenylsuccinate trade name material (PS-111 hydrophobically modified starch powder)	*Salmonella typhimurium* strains TA98, TA 100, TA 1535, and TA 1537; *E. coli* strain WP2*uvr*A	Ames test	up to 5,000 µg/plate, with and without metabolic activation	Not genotoxic.[181]
Stearoyl inulin (Rheopearl ISK2 and Rheopedarl ISL2)	*Salmonella typhimurium* and *E. coli* (strains not stated)	Ames test	Doses and presence/absence of activation not stated	Not genotoxic.[71,72]
	Mammalian Assays			
Linear polysaccharides and their salts				
PNG or Refined Carrageenan	Bone marrow cells from Swiss mice	Micronucleus test	Mice received PNG at doses up to 2,500 mg/kg body weight or refined carrageenan at a dose of 700 mg/kg body weight	Neither PNG nor refined carrageenan was genotoxic.[179]
Branched - modified (i.e., added sidechains are larger than acetate)				
Carboxymethyl inulin	Chinese hamster ovary (CHO-WBL) cells	Chromosome aberrations assay	up to 5,060 µg/ml, with and without metabolic activation	No significant increases in chromosomal aberrations, polyploidy, and endoreduplication.[156]

Table 10. Genotoxicity of Polysaccharide Gums

Ingredient/Similar Chemical	Strain/cell type	Assay	Dose	Results
Potato starch modified	Mice (strain not stated)	Mouse lymphoma assay. OECD 476 test guideline.	Not stated	Not genotoxic.[155]
Branched - unmodified				
Ghatti gum	Chinese hamster ovary (CHO-WBL) cells	Chromosome aberrations assay	up to 6,000 µg/ml, with and without metabolic activation	Not genotoxic.[180]
Ghatti gum	B6C3F1 mice	Combined micronucleus/Comet assay	Mice dosed orally with up to 2,000 mg/kg/day for 4 days	No effect on micronucleated reticulocyte frequency in peripheral blood. No DNA damage in blood leukocytes or liver.[180]
Glucomannan	L5178Y $tk^{+/-}$ mouse lymphoma cells	Mouse lymphoma assay	Up to 1,000 µg/ml and up to 997 µg/ml with and without metabolic activation, respectively	Not genotoxic.[165]
Glucomannan	CD-1 (ICR) mouse bone marrow cells	Micronucleus test	Mice dosed orally with 5,000 mg/kg body weight	Not genotoxic.[165]
Pectin-derived acidic oligosaccharides (for genotoxicity evaluation of Pectin)	L5178Y mouse lymphoma cells	Mouse lymphoma assay	up to 4370 µg/ml, with and without metabolic activation	Equivocal results.[168]
Pectin-derived acidic oligosaccharides (for genotoxicity evaluation of Pectin)	Chinese hamster ovary cells	Chromosome aberrations assay	up to 4,220 µg/ml, with and without metabolic activation	Clastogenic. Dose-related genotoxicity at ≥ 2,530 µg/ml without metabolic activation. Positive results only at highly cytotoxic concentrations.[168]
Pectin-derived acidic oligosaccharides (for genotoxicity evaluation of Pectin)	F_1 rats (from outbred strain of Wistar rats (Crl:WI(WU))	Micronucleus test	Oral administration of diet containing pectin-derived acidic oligosac-charides (pAOS) (±7 g/kg body weight/day) for 13 weeks.	Compared to control, no increase in mean number of micronuclei in rat erythrocytes.[168]
Sterculia urens gum	Sprague-Dawley rats	Cytogenetic assay	Groups of rats intubated with 5,000 mg/kg, 2500 mg/kg, and 30 mg/kg, respectively. Metapase chromo-somes from rat bone marrow analyzed.	No adverse effect on rat bone marrow chromosomes.[153]
Sterculia urens gum	WI-38 human embryonic lung cells	Cytogenetic assay	up to 1,000 µg/ml	No effect on anaphase chromosomes.[153]

Table 10. Genotoxicity of Polysaccharide Gums

Ingredient/Similar Chemical	Strain/cell type	Assay	Dose	Results
Sterculia urens gum	Sprague-Dawley rats	Dominant lethal gene test	Groups of rats intubated with 5,000 mg/kg, 2500 mg/kg, and 30 mg/kg, respectively	No consistent responses suggestive of genotoxicity.[153]
Wheat bran extract (contains ~ 80% arabinoxylan) (for genotoxicity evaluation of Arabinoxylan)	Chinese hamster lung fibroblasts	Chromosome aberrations assay	up to 5,000 µg/ml, with and without metabolic activation	Not genotoxic or clastogenic.[163]

Table 11. Carcinogenicity of Polysaccharide Gums

Oral

Linear Polysaccharides and Their Salts

Agar: 25,000 ppm or 50,000 ppm agar. Groups of 50 F344 rats and 50 B6C3F1 mice of each sex. Feeding in diet for 103 weeks. Untreated mice and rats served as controls. No clinical signs of toxicity. Increased incidence (not statistically significant) of adrenal cortical adenomas in female rats fed 50,000 ppm agar. Statistically significant increase ($p = 0.007$) in incidence of hepatocellular adenomas in male mice fed 50,000 ppm agar. Incidence of total liver tumors did not differ statistically among control, 25,000 ppm, and 50,000 ppm groups. Increased incidences of adrenal cortical adenomas and liver tumors not considered test substance-related. Agar was non-carcinogenic.[182]

Algin: Up to 25% sodium alginate. Mice (75 males; 75 females). Feeding in diet for 89 weeks (dietary levels gradually increased to maximum concentration of 25%). At week 87, half of surviving male and female mice placed on control diet containing 55% pregelatinized potato starch. Algin was non-carcinogenic.[162]

Carrageenan: 5% ι-carrageenan. Groups of 16 Fischer 344 rats. Feeding for up to 91 days. Proliferating cell nuclear antigen (PCNA) served as a marker of cell proliferation. Immunohistochemical staining for PCNA-positive cells in distal colon performed. Intact layer of columnar epithelial cells lining the mucosa. PCNA-positive cells not found at the luminal surface.[183]

Carrageenan: 0.5%, 1.5%, and 5% ι-carrageenan. Groups of four F344 rats. Feeding in diet for 28 days. Control diet fed to additional group. Thymidine kinase enzymatic activity and PCNA served as markers of cell proliferation. No increase in PCNA-positive cells. Increased thymidine kinase levels observed only in the 5% ι-carrageenan dietary group, corresponding to a 4-fold increase in colonic cell proliferation.[183]

Carrageenan: ι-carrageenan. F344 rats. Feeding in diet for 64 days, followed by 28-day recovery period. During recovery period, proliferating cells returned to level similar to those in rats fed control diet. Results suggest that the quantitative changes in cell proliferation were probably adaptive, and would not contribute to an increased risk of colon neoplasia.[183]

Carrageenan: 0.1, 5, 15, and 25% carrageenan. Groups of 5 male and 5 female mice of two strains. Feeding in the diet for lifespan. Additional group fed control diet. Non-carcinogenic.[184]

Carrageenan: 1, 5, 15, and 25% carrageenan. Groups of 5 male and 5 female mice of two strains. Feeding in diet for up to 24 months. Additional group fed control diet. Hepatic sclerosis at 25% concentration. Non-carcinogenic.[184]

Carrageenan: 0.5, 2.5, and 5% κ-carrageenan. MRC outbred rats and randomly bred Syrian golden hamsters from the Eppley colony (30 males and 30 females per species). Average daily intake of carrageenan estimated to be 4022 mg/kg/day (rats) and 3719 mg/kg/day (hamsters) for lifetime. 100 females and 100 males per control dietary group. No increased mortality, clinical signs of toxicity, or tumor formation.[185]

Carrageenan: Groups of female Fischer 344 rats. Co-carcinogenicity of carrageenan in presence of azoxymethane (AOM) or N- nitrosomethylurea (NMU) evaluated. Treatment groups: control diet (15 rats); 15% carrageenan in control diet (15 rats); 15% carrageenan in control diet + 10 weekly s.c. injections of 8 mg/kg bw (AOM) (30 rats); 2 mg NMU (intrarectal instillations) twice weekly for 3 weeks (30 rats); AOM s.c. alone (30 rats), and NMU i.r. alone (30 rats). Animals killed 40 weeks after the initial injection of AOM or 30 weeks after the initial injection of NMU. Carrageenan enhanced the incidence of colon tumors in AOM- and NMU-treated rats ($p < 0.01$): AOM + carrageenan (26/26, 100%) versus AOM alone (17/30, 57%); NMU + carrageenan (29/29, 100%) versus NMU alone (20/29, 69%); control diet (0/15); and 15% carrageenan in control diet (1/15, 7%).[186]

Carrageenan: Carrageenan (0.25%, 2.5%, or 10%). Aberrant crypt focus (ACF) assay for assessment of initiation and promotion of cancer. 24 rats randomly allocated to 3 groups in initiation experiment: 9 rats given carrageenan (as a 10% jelly [24.7 g/kg body weight per day] for 8 days) in initiation experiment, 9 rats were given pure water (negative controls), and 6 rats received AOM injection (5 mg/kg i.p., positive controls). Promotion experiment: 30 rats received single azoxymethane injection (20 mg/kg i.p.) to initiate colon cancer. Seven days later, the rats were randomly allocated to the following 3 groups of 10: control group (received distilled water), group 1 (received water supplemented with 0.25% carrageenan [liquid] for 100 days), and group 2 (received water supplemented with 2.5% carrageenan [solid gel] for 100 days). In initiation experiment, no ACF found in negative controls or in rats fed carrageenan. In promotion experiment, administration of liquid 0.25% carrageenan reduced number of ACF/rat, and did not change the ACF multiplicity when compared to controls. In contrast, administration of carrageenan jelly (2.5%) for 100 days promoted growth of aberrant crypt foci ($P = 0.016$). Thus, carrageenan jelly did not initiate colon tumors; however, long-term administration of carrageenan jelly enhanced intestinal tumor growth in rats.[187]

Carrageenan: κ-carrageenan (0.5%, 2.5%, or 10%). 54 conventional female Fischer 344 (F-344) rats (harboring a normal rat flora) and 52 germ-free female F-344 rats maintained in isolators. Intiating effect of κ-carrageenan studied by comparing number of ACF in the colon of rats given pure water or κ-carrageenan (as a 10% gel in tap water) for 8 days. Promoting effect of κ-carrageenan studied by comparing multiplicity of ACF (crypts/ACF) in rats that received pure water, liquid κ-carrageenan (0.25% in water), or κ-carrageenan gel (2.5% in water) during 100 days, beginning 7 days after a single AOM injection. κ-carrageenan (10%) did not initiate ACF. In conventional rats, the 2.5% κ-carrageenan gel promoted the growth of ACF as follows: 2.98 ± 0.29 and 3.44 ± 0.48 crypts/AF in control and treated rats, respectively ($p < 0.02$). 0.25% κ-carrageenan gel did not promote ACF.[188]

Carrageenan: 2.5% κ-carrageenan. 8 HFA ratsgiven κ-carrageenan and an additional 8 given water; 4 rats received AOM injection. No promotion effect: $2.81 \pm 0.1$and 2.78 ± 0.38 crypts/ACF in control and treated rats, respectively ($p = 0.80$).[188]

Carrageenan: Carrageenan (1.25%, 2.5%, or 5.0%). Groups of 18 rats or 6 rats. Groups of 18 initiated with DMH, followed by feeding with 1.25%, 2.5%, or 5% in diet for 32 weeks. Groups of 6 received saline and were then treated with 0% and 5.0% carrageenan. Detailed histopathological examination did not demonstrate any carrageenan-induced enhancement of carcinogenesis. Thus, carrageenan did not possess any promoting activity for colorectal carcinogenesis at any dietary concentration.[189]

Carrageenan: In a monograph published by the International Agency for Research on Cancer (IARC) in 1983, IARC concluded that the available data do not provide evidence that native (undegraded) carrageenan is carcinogenic to experimental animals, and, in the absence of epidemiological data, that no evaluation of the carcinogenicity of native carrageenan in humans could be made.[92]

Inulin: Inulin-enriched diet (10% w/w). Group of 10 to 15 Min/+ mice (has heterozygous mutation in the Apc gene, resulting in the truncated Apc protein and development of numerous intestinal adenomas.[190,191]) fed from the age of 5 weeks to 8 or 15 weeks. Additional group fed control diet. Results indicated that dietary inulin can activate mucosal β-catenin signaling, which, in the presence of Apc mutation, induces adenoma growth.[192]

Table 11. Carcinogenicity of Polysaccharide Gums

Inulin: 3 Groups of 10 Sprague-Dawley rats, consisting of control group, group treated s.c.with DMH, and group given DMH and inulin in the diet. When compared to the DMH only group, inulin in diet decreased the expression of IL-2, TNFα, and IL-10 and also decreased the numbers of COX-2- and NFκB-positive cells in the *tunica mucosae* and *tela submucosae* of the colon. Thus, dietary intake of inulin prevented preneoplastic changes and inflammation that promote colon cancer development.[193]

Inulin: Inulin (15 g) in basal diet (85 g). Groups of 20 to 22 Balb/c mice. Feeding for 7 days prior to tumor (TLT and EMT6 tumor cell lines) transplantation. Growth of both tumor cell lines significantly inhibited by supplementing the diet with inulin.[194]

Branched - Unmodified

Arabinoxylan: Groups of 15 rats treated (s.c.) with the colon carcinogen DMH and fed either a control diet or a diet containing arabinoxylan-oligosaccharides (4.8% w/w). Two types of preneoplastic lesions (ACF and mucin-depleted foci [MDF]) detected in colon. Thirteen weeks after DMH treatment, MDF counts significantly lower in entire colon of arabinoxylan-oligosaccharides fed rats (MDF/colon were 7.5 ± 0.6 and 5.5 ± 0.6, in control and arabinoxylan-oligosaccharides groups, respectively; means ± SE [p = 0.05]). Arabinoxylan-oligosaccharides fed rats had significantly fewer ACF in the distal part of the colon than control rats (ACF/distal colon were 135.5 ± 15 and 84.4 ± 11, in control and arabinoxylan-oligosaccharides groups, respectively; means ± SE [p = 0.05]). Thus, dietary intake of arabinoxylan-oligosaccharides by rats reduced the occurrence of two types of preneoplastic lesions, suggesting a chemopreventive effect on colon carcinogenesis.[195]

Arabinoxylan: Groups of 10 ICR male mice. mice were injected i.p. with mouse sarcoma S180 cells, human chronic myelogenous K562 cells, or human leukemia HL-60 cells, and dosed orally with arabinoxylan (100, 200, or 400 mg/kg body weight). All three doses conferred significant inhibitory activity against solid tumor formation in S180 tumor-bearing mice, with inhibitory ratios of 14.34%, 31.37%, and 56.73%, respectively. Arabinoxylan did not have any effect on growth of K562 or HL-60 cells in *vitro*.[196]

Glucomannan: 10% Glucomannan. Groups of 30 C3H/He male mice fed either a powdered commercial diet (control group) or the same diet containing 10% glucomannan. At age 1 year, slight decrease in the number of animals with liver tumors in glucomannan group (control: 63% of 24 mice; glucomannan: 48% of 23 mice) and a statistically significant decrease ($p<0.05$) in the mean number of tumor nodules per mouse in the glucomannan group (control: 1.1; konjac mannan: 0.5). Thus, spontaneous liver tumors in C3H/He mice were inhibited by maintaining the mice on a diet containing 10% glucomannan.[197]

Glucomannan: 5% Glucomannan. Fisher 344 rats (20/group) fed either a commercial diet or similar diet containing 5%glucomannan for 13 weeks. Animals also injected i.p. with DMH weekly. Incidence of DMH-induced colon tumors significantly lower in glucomannan-fed group (39%) when compared to control group (75%). Number of colon adenocarcinomas per rat also significantly lower in glucomannan-fed rats (0.22) than in control rats (0.75). No significant effect on the incidence of tumors of the small intestine, all of which were adenocarcinoma (control: 45%; konjac mannan: 33%).[198]

Pectin: 2.5% Pectin. Male Wistar rats (groups of 4). Feeding in diet for 14 days. Statistically significant increase in the villus height and crypt depth, indicating that feeding with pectin caused mucosal hyperplasia in small intestine.[199]

Starch Acetate: 55% Starch Acetate. Mice (75 males ,75 females) fed starch acetate in diet for 89 weeks. Dietary levels of the test substance gradually increased until diet contained (by weight) 55% starch acetate. At week 87, half ofsurviving male and female mice placed on control diet (containing 55% pregelatinized potato starch). No evidence of carcinogenicity.[162]

Cyclic

Cyclodextrin: 2.5% or 5% β-cyclodextrin. 2 groups of Fischer 344 (F344) rats (50 males and 50 females/group) fed 2.5%and 5% β-cyclodextrin, respectively, for 104 weeks. Control diet fed to additional group. All neoplastic lesions observed were histologically similar to those known to occur spontaneously in this strain of rat; no statistically significant increase in the incidence of any tumor found for either sex in treated groups. It was concluded that the high dose, which was approximately 340-400 times higher than the current daily human intake from ingestion as a food additive and from pharmaceutical use, did not have carcinogenic potential in F344 rats.[111]

Cyclodextrin: β-cyclodextrin . 5 groups of 50 Fischer 344 rats and 52 CD-1 outbred mice of each sex. 4 groups per strain received β-cyclodextrin in the diet at doses of 25, 75, 225, and 675 mg/kg per day, respectively for 93 weeks (males) and between weeks 129 and 130 (females). Fifth group received control diet. No treatment-related carcinogenic effects.[200]

Degraded Polysaccharide Gum

Degraded Carrageenan: Degraded carrageenan (from *Eucheuma spinosum*; degraded by acid hydrolysis). 4 groups of 30 malesand 30 female rats 8fed a diet containing 0 (control), 1 %, 5 %, or 10% degraded carrageenan. Colorectal squamous metaplasia in rats fed degraded carrageenan at concentrations of 10% (59 of 60 rats) and 5% (53 of 60 rats) in the diet. Additionally, colorectal tumors (12 squamous-cell carcinomas, 8 adenocarcinomas and 3 adenomas) found in 19 of 60 rats fed 10% degraded carrageenan in the diet, and these tumors (3 squamous-cell carcinomas, 1 adenocarcinoma and 8 adenomas) also found in 12 of 60 rats fed 5% degraded carrageenan. Neither squamous metaplasia nor colorectal tumors observed in the low-dose group or in controls.[92]

Degraded Carrageenan: Degraded carrageenan (5% in drinking water) administered to 20 male and 20 female rats for 15 months. Colorectal squamous metaplasia observed in all rats after 15 months. Colorectal tumors observed in 11 of 40 treated rats (4 squamous-cell carcinomas, 4 adenocarcinomas, 3 adenomas and 1 myosarcoma); these tumors not observed in control rats (15 males, 15 females).[201]

Degraded Carrageenan: Degraded carrageenan (1 or 5 g/kg body weight) administered by intragastric intubation (frequency of administration not specified) to groups of 15 male and 15 female rats for 15 months. Control rats (15 males, 15 females) dosed intragastrically with distilled water. Squamous colorectal metaplasia observed in all 29 rats in high-dose group and in 11 of 30 rats in low-dose group. Colorectal tumors were observed only in the high-dose group (8 of 29 rats; 5 adenocarcinomas and 4 adenomas).[202]

Degraded Carrageenan 10% degraded carrageenan (in diet that also contained 30% sulfate) fed to Fischer 344 rats. Three groups fed this diet for 2 months (39 rats, group 1), 6 months (42 rats, group 2), and 9 months (42 rats, group 3). Control group (46 rats) received the same diet without carrageenan, and the same was true for all other groups after cessation of feeding. 100% incidence of colorectal squamous metaplasia observed in all treatment groups. Tumors also observed in 5 of 39 rats in group 1 (3 squamous-cell carcinomas, 1 adenoma, 1 anaplastic carcinoma), 8 of 42 rats in group 2 (6 squamous-cell carcinomas, 1 adenocarcinoma, 1 adenoma) and in 17 of 42 rats in group 3 (14 squamous-cell carcinomas, 4 adenocarcinomas). Colorectal changes not observed in control rats.[92,203]

Table12. Skin Irritation/Sensitization Potential of Polysaccharide Gums

Skin Irritation and Sensitization - Non-Human

Linear Polysaccharides and Their Salts

Algin: 2% algin. Rabbits (number not stated). 3 primary skin irritation experiments. Occlusive patches applied to the skin. Mean skin irritation score of < 0.5 = non-irritating; 0.5 to 2.0 = slightly irritating. Primary irritation index (PII) values calculated. PII of < 0.5 deemed satisfactory, but PII no greater than 1 is also acceptable. PII values of 0, 0, and 0.08 were reported in the 3 experiments, respectively.[61]

Algin: 2% algin. Rabbits (3 per experiment). Test substance (2 ml) applied to flanks 5 days per week for 6 weeks. Mean maximum irritation index (MMII) values calculated. Macroscopic and histological examinations of test sites performed. MMII values of 0.67, 0, and 0.67 were reported in 3 experiments, respectively. Daily application of test substance did not induce a severe reaction at either macroscopic or histological examination.[61]

Carrageenan: Food grade iota-carrageenan (one subtype of carrageenan with a specific number and position of sulfate groups on the repeating galactose units). Guinea pigs (number not stated). Study details not included. No skin sensitization.[62]

Branched - Unmodified

Glucomannan (in konjac flour [mechanically ground]). Guinea pigs (number not stated). Application to skin according to the Buehler closed patch method. No sensitization.[151]

Branched - Modified

Corn Starch Modified: Corn starch modified in distilled water (30% solids). 10 Zealand White rabbits (5 males and 5 females). Application to skin (2,000 mg/kg); dose per cm^2 not stated. Dermal reactions either absent or classified as barely perceptible at 24-h and 48-h readings, and absent at the 74-h reading. Mild skin irritant (primary irritation index = 0.25).[66]

Corn Starch Modified: Corn Starch Modified (up to 30%). 20 guinea pigs (strain not stated; 10 males, 10 females). Maximization test (OECD protocol 406.) During induction,10% solution injected and 30% solution applied topically. Concentration per cm^2 was not stated. During challenge, application of 20% solution for 24 h. Reactions scored at 48 h and 72 h post-application. Control group (5 males, 5 females) tested with distilled water during induction and challenged with test substance. Reactions ranging from no erythema to moderate erythema observed after induction with the control or test substance. Erythema observed after challenge with test substance. However, rechallenge with same test substance concentration did not cause erythema. Not a sensitizer.[66]

Corn Starch Modified: 50% corn starch modified paste. 25 female Hartley guinea pigs. RIPT according to Buehler method (OECD protocol 4067). 10 guinea pigs treated with distilled water (control). Positive control (isoeugenol) tested in study performed within 6 months of current study. During induction, test material applied topically to shoulder area (~ 0.4 g on occlusive patch; area of application site not stated) . Topical challenge with 50% corn starch modified paste for 6 h. Challenge reactions scored at 24 h and 48 h post-application. No erythema or edema during induction or challenge. Non-sensitizer. Positive control induced sensitization.[63]

Carboxymethyl Inulin: Carboxymethyl inulin (1% to 100%). Groups of 2 adult Dunkin–Hartley albino guinea pigs. Test substance injected into clipped scapular region; reactions scored at 24 h and 48 h. Also, series of test article concentrations (0.5 ml) applied topically for 24 h to clipped external flank using Metalline patches secured with tape and an elastic bandage. Test material was removed after 24 h and signs of irritation recorded at 24 h and 48 h after treatment. Undiluted carboxymethyl inulin produced necrosis after intradermal injection, observed both after 24 h and 48 h; 20% to 50% did not cause necrosis, but grade 2 erythema was observed at either 24 h or 48 h. Signs of irritation were not observed at 24 h or 48 h at concentrations up to 100% in the patch tests.[156]

Carboxymethyl Inulin: 31.1% aqueous carboxymethyl inulin. 10 adult Dunkin–Hartley albino guinea pigs. Maximization test. Five female guinea pigs served as vehicle controls. No evidence of sensitization.[156]

Potato Starch Modified: 10 rats received single dose of potato starch modified (dose = 2 g/kg) dermally. Very slight to well-defined erythema and edema observed in all animals after 24 h. At 48 h, very slight erythema and very slight edema in 5 and 3 rats, respectively. All reactions had cleared by 72 h.[155]

Potato Starch Modified: Rats (10 males, 10 females). Dose of 2 g/kg body weight/day applied to the skin, under occlusive dressing, for 28 days . Neither erythema nor edema observed. However, small scabs observed on 5 males and 6 females, attributed to adhesion of test material to skin.[155]

Potato Starch Modified: Potato starch modified (18.5% aqueous suspension). 20guinea pigs. Buehler test (OECD 406 test guideline). Faint erythema (non-confluent) observed in 6 of 20 animals after second or third induction application. No evidence of sensitization.[155]

Potato Starch Modified: Potato Starch Modified (10% solids aqueous solution). 10 male and 10 female New Zealand albino rabbits (test animals)). Using non-occlusive patch, test substance (2 g/kg body weight) applied to the skin. The area of application and dose per cm^2 not stated. 20 control animals tested with distilled water under non-occlusive patch. Neither erythema nor edema observed in treated or control animals. No adverse morphologic effects on the skin.[70]

Potato Starch Modified: Potato starch modified (18.5% solids). 20 guinea pigs (10 males, 10 females). RIPT according to Buehler method (OECD 406 protocol). Concentration per cm^2 not stated. 10 control animals (5 males, 5 females) treated with distilled water. During induction, very faint erythema in 6 of 20 animals; reactions not observed in controls. Very faint erythema observed in 2 of 20 treated animals and in 2 of 10 controls during challenge phase. Non-sensitizer.[70]

Dextrin Myristate: 6 New Zealand white rabbits. Skin irritation study (test protocol not stated). Non-irritant.[67]

Dextrin Myristate: Guinea pigs (number and strain not stated). Magnusson-Kligman maximization test. No evidence of skin sensitization.[67]

Dextrin Palmitate: 3 New Zealand white rabbits. Skin irritation study (test protocol not stated). Non-irritant.[68,69]

Table12. Skin Irritation/Sensitization Potential of Polysaccharide Gums

Branched - Modified

Dextrin Palmitate: Guinea pigs (number and strain not stated). Magnusson-Kligman maximization test (test concentrations not stated). No evidence of skin sensitization.[68,69]

Sodium Hydrolyzed Potato Starch Dodecenylsuccinate: Test Material: Material (corn starch modified) described as structurally similar to sodium hydrolyzed starch dodecenylsuccinate and as the calcium salt of the ester formed from the reaction of 3-(dodecenyl)dihydro-2,5-furandione and corn starch, in which the degree of substitution per glucose unit is less than 0.1. 6 New Zealand White rabbits. OECD 404 test protocol. 50% slurry of test material (1 ml) applied topically (on occlusive patch, area of application site not stated) for 24 h to intact and abraded skin sites on the back of each animal. Reactions scored for up to 72 h after patch application. Erythema observed at intact and abraded sites on one animal, and reactions had cleared by 48 h. Mildly irritating to the skin (primary irritation index = 0.09).[63,204]

Stearoyl Inulin: 6 Japanese white rabbits. Skin irritation potential evaluated (concentrations and test protocol not stated). Non-irritant.[71,72]

Stearoyl Inulin: Guinea pigs (number and strain not stated). Skin sensitization potential evaluated (concentrations not stated) according to adjuvant and patch method. Skin irritation classified as weak. Very low skin sensitization potential.[71,72]

Skin Irritation and Sensitization - Human

Linear Polysaccharides and Their Salts

Algin: 20% aqueous sodium alginate. 12 male subjects with no history of allergy. Patch-testing (Finn chambers) with 20% aqueous sodium alginate according to International Contact Dermatitis Research Group (ICDRG) recommendations. Area (cm^2) of application and dose per cm^2 not stated. Reactions scored at 2 and 3 days post-application. ± reaction observed in one subject on days 2 and 3. Results negative for skin irritation and allergic contact dermatitis.[205]

Linear - Modified

Hydrolyzed Furcellaran: Mixture containing 1.35% furcellaran powder and 1% phenoxyethanol. 10 adults. Mixture applied (under occlusive patch) for 48 h to back. Area (cm^2) of application and dose per cm^2 not stated. Non-irritant.[73]

Hydrolyzed Furcellaran: Mixture containing 1.35% furcellaran powder, 0.1% potassium sorbate, and 0.05% citric acid. 10 adults. Mixture applied (under occlusive patch) for 48 h to back. Area (cm^2) of application and dose per cm^2 not stated. Non-irritant and non-sensitizer.[73]

Hydrolyzed Furcellaran: Mixture containing 0.6% hydrolyzed furcellaran, 0.05% concentrate of sea water, 1% phenoxyethanol, and 98.35% water. 100 subjects. Mixture applied 9 times to each subject. Area (cm^2) of application and dose per cm^2 not stated. Non-irritant and non-sensitizer.[73]

Maltodextrin: Eye gel containing 2.45% maltodextrin. 103 subjects. HRIPT. Patch type, area (cm^2) of application, and dose per cm^2 not stated. Challenge patches applied to original and alternate sites, and challenge reactions scored at approximately 48 h and 96 h post-application. Five instances of erythema (grade 1) during induction. At 48-h challenge reading, a grade of 1 reported for alternate challenge site of one subject. Gel did not induce allergic contact dermatitis.[206]

Branched - Modified

Corn Starch Modified: 7.5% solution in distilled water. 26 female subjects. 21-day cumulative irritation study. Test material (0.2 ml per 24-h patch) applied topically. Area (cm^2) of application and dose per cm^2 not stated. Reactions ranged from no erythema to minimal erythema. Non-irritant. Distilled water (vehicle control) did not cause erythema. Sodium lauryl sulfate (positive control) induced marked erythema and papules.[66]

Corn Starch Modified: 7.5% solution in distilled water. 113 subjects (86 females, 27 males). HRIPT. Patch type, area (cm^2) of application, and dose per cm^2 not stated. Challenge eactions scored at 48 h and 96 h post-application. Test substance and distilled water caused slight erythema in 3 subjects. Test substance and distilled water classified as non-sensitizers.[66]

Dextrin: Rinse-off facial product containing 42.6919 % dextrin (1% aqueous; effective concentration ≈ 0.4%). 54 subjects (46 females, 8 males). HRIPT. During induction, product (0.1-0.15 g on occlusive patch) applied for 24 h to the back.. Dose/concentration per cm^2 not stated. Challenge patch applied to new test site and reactions scored at 24 h and 72 h post-application. Transient, barely perceptible erythema, in 1 subject, during induction. No reactions observed during challenge phase. No clinically significant skin irritation or evidence of allergic contact dermatitiss.[207]

Dextrin Myristate: Leave-on facial product containing 0.3% dextrin myristate. 51 subjects (40 females, 11 males). HRIPT. During induction, product (0.1-0.15 g on occlusive patch) applied for 24 h to the back.. Dose/concentration per cm^2 not stated. Challenge patch applied to new test site and reactions scored at 24 h and 72 h post-application. Skin reactivity was not observed during the induction or challenge phase. Product did not cause skin irritation or allergic contact dermatitis.[208]

Hydroxypropyltrimonium Hydrolyzed Corn Starch: 15% hydroxypropyltrimonium hydrolyzed corn starch. 47 male and female subjects. HRIPT. During induction, semi-occlusive patch (1" x 1") containing approximately 0.2 ml of test material applied for 24 h to upper back. 24-h challenge patch applied to new test site, adjacent to induction patch site. No reactions during study. No skin irritation or allergic contact sensitization potential.[209]

Calcium Starch Isododecenylsuccinate: Test material (powder) and a 50% w/v slurry of test material in baby oil tested. 23 subjects. Powder applied topically (0.2 g, moistened with distilled water; area of application site not stated) under occlusive conditions for 21 days. 50% w/v slurry applied acccording to same procedure. Powder caused dermal effects that ranged from no irritation to erythema and papules cumulative irritation score = 177). Superficial layer effects ranged from none to glazing with peeling and cracking. 50% w/v slurry caused milder reactions (cumulative irritation score = 50.6). Both test materials classified as probable mild irritants under normal use conditions.[63,64,210]

Branched - Modified

Sodium Hydrolyzed Potato Starch Dodecenylsuccinate: Cleanser containing 10 wt% sodium hydrolyzed potato starch dodecenylsuccinate. 227 subjects (18 to 69 years old; 165 females, 62 males). HRIPT. During induction, occlusive patch containing ~ 0.2 g of the test material was applied to the back (area of application site not stated) for 24 h. week non-treatment period. Occlusive challenge patch containing the test material (~ 0.2 g) applied for 24 h to new site on back. Reactions were scored for up to 96 h post-application. Four subjects had low-level (±) reactions during induction, and 2 subjects had ± reactions during challenge phase. Non-sensitizer.[211]

Table12. Skin Irritation/Sensitization Potential of Polysaccharide Gums

Unknown Structural Configuration

Algae Exopolysaccharides: 1% solution of algae exopolysaccharides. 50 subjects. HRIPT. During induction, occlusive patch containing test substance (0.2 ml or 0.2 g) applied for 24 h to infrascapular region of back. Dose per cm^2 not stated. Challenge dose (equivalent to induction application) of test substance applied once to new test site. Reactions scored at 24 h to 48 h post-application. No evidence of adverse reactions. Not a primary skin irritant or sensitizer.[212]

In Vitro

Branched - Modified

Hydroxypropyltrimonium Hydrolyzed Corn Starch: MatTek Corporation EpiDerm™ skin model *in vitro* toxicity testing system. Skin model consists of normal, human-derived epidermal keratinocytes (NHEK) that have been cultured to form a multilayered, highly differentiated model of the human epidermis. Test procedure utilizes a water-soluble, yellow tetrazolium salt MTT. In the mitochondria of viable cells, MTT is reduced by succinate dehydrogenase to an insoluble formazan derivative (purple color). Substances that damage this enzyme inhibit reduction of the tetrazolium salt. Undiluted test substance (100 µl) added to millicells containing EpiDerm™ samples; time at which % viability would be 50% (ET_{50}) estimated. Mild irritant (ET_{50} = 18.1h).[213]

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SAFETY DATA SHEET

according to Regulation (EC) No. 1907/2006

Version 6.3
Revision Date 25.04.2022
Print Date 08.10.2022

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1 Product identifiers

Product name	:	Carrageenan Carrageenan
Product Number	:	C1013
Brand	:	Sigma
REACH No.	:	A registration number is not available for this substance as the substance or its uses are exempted from registration, the annual tonnage does not require a registration or the registration is envisaged for a later registration deadline.
CAS-No.	:	9000-07-1

1.2 Relevant identified uses of the substance or mixture and uses advised against

Identified uses	:	Laboratory chemicals, Manufacture of substances

1.3 Details of the supplier of the safety data sheet

Company	:	Merck Life Science UK Limited New Road The Old Brickyard GILLINGHAM Dorset SP8 4XT UNITED KINGDOM
Telephone	:	+44 (0)1747 833-000
Fax	:	+44 (0)1747 833-313
E-mail address	:	TechnicalService@merckgroup.com

1.4 Emergency telephone

Emergency Phone #	:	+44 (0)870 8200418 (CHEMTREC)

SECTION 2: Hazards identification

2.1 Classification of the substance or mixture

Not a hazardous substance or mixture according to Regulation (EC) No 1272/2008.

2.2 Label elements

Not a hazardous substance or mixture according to Regulation (EC) No 1272/2008.

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2.3 Other hazards

This substance/mixture contains no components considered to be either persistent, bioaccumulative and toxic (PBT), or very persistent and very bioaccumulative (vPvB) at levels of 0.1% or higher.

SECTION 3: Composition/information on ingredients

3.1 Substances

Synonyms	:	Vegetable gelatin
CAS-No.	:	9000-07-1
EC-No.	:	232-524-2

No components need to be disclosed according to the applicable regulations.

SECTION 4: First aid measures

4.1 Description of first-aid measures

If inhaled
If breathed in, move person into fresh air. If not breathing, give artificial respiration.

In case of skin contact
Wash off with soap and plenty of water.

In case of eye contact
Flush eyes with water as a precaution.

If swallowed
Never give anything by mouth to an unconscious person. Rinse mouth with water.

4.2 Most important symptoms and effects, both acute and delayed

The most important known symptoms and effects are described in the labelling (see section 2.2) and/or in section 11

4.3 Indication of any immediate medical attention and special treatment needed

No data available

SECTION 5: Firefighting measures

5.1 Extinguishing media

Suitable extinguishing media
Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

5.2 Special hazards arising from the substance or mixture

Nature of decomposition products not known.

5.3 Advice for firefighters

Wear self-contained breathing apparatus for firefighting if necessary.

5.4 Further information

No data available

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SECTION 6: Accidental release measures

6.1 Personal precautions, protective equipment and emergency procedures
Avoid dust formation. Avoid breathing vapors, mist or gas.
For personal protection see section 8.

6.2 Environmental precautions
No special environmental precautions required.

6.3 Methods and materials for containment and cleaning up
Sweep up and shovel. Keep in suitable, closed containers for disposal.

6.4 Reference to other sections
For disposal see section 13.

SECTION 7: Handling and storage

7.1 Precautions for safe handling

Advice on protection against fire and explosion
Provide appropriate exhaust ventilation at places where dust is formed.

Hygiene measures
General industrial hygiene practice.
For precautions see section 2.2.

7.2 Conditions for safe storage, including any incompatibilities

Storage conditions
Keep container tightly closed in a dry and well-ventilated place. Store in cool place.

Storage class
Storage class (TRGS 510): 13: Non Combustible Solids

7.3 Specific end use(s)
Apart from the uses mentioned in section 1.2 no other specific uses are stipulated

SECTION 8: Exposure controls/personal protection

8.1 Control parameters

Ingredients with workplace control parameters
Contains no substances with occupational exposure limit values.

8.2 Exposure controls

Personal protective equipment

Eye/face protection
Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU).

Skin protection
Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

The selected protective gloves have to satisfy the specifications of Regulation (EU) 2016/425 and the standard EN 374 derived from it.

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Body Protection
Choose body protection in relation to its type, to the concentration and amount of dangerous substances, and to the specific work-place., The type of protective equipment must be selected according to the concentration and amount of the dangerous substance at the specific workplace.

Respiratory protection
Respiratory protection is not required. Where protection from nuisance levels of dusts are desired, use type N95 (US) or type P1 (EN 143) dust masks. Use respirators and components tested and approved under appropriate government standards such as NIOSH (US) or CEN (EU).

Control of environmental exposure
No special environmental precautions required.

SECTION 9: Physical and chemical properties

9.1 Information on basic physical and chemical properties

a)	Physical state	solid
b)	Color	No data available
c)	Odor	No data available
d)	Melting point/freezing point	No data available
e)	Initial boiling point and boiling range	No data available
f)	Flammability (solid, gas)	May form combustible dust concentrations in air.
g)	Upper/lower flammability or explosive limits	No data available
h)	Flash point	No data available
i)	Autoignition temperature	No data available
j)	Decomposition temperature	No data available
k)	pH	No data available
l)	Viscosity	Viscosity, kinematic: No data available Viscosity, dynamic: No data available
m)	Water solubility	No data available
n)	Partition coefficient: n-octanol/water	No data available
o)	Vapor pressure	No data available
p)	Density	No data available
	Relative density	No data available
q)	Relative vapor density	No data available
r)	Particle	No data available

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characteristics

s) Explosive properties No data available

t) Oxidizing properties No data available

9.2 Other safety information
No data available

SECTION 10: Stability and reactivity

10.1 Reactivity
No data available

10.2 Chemical stability
Stable under recommended storage conditions.

10.3 Possibility of hazardous reactions
No data available

10.4 Conditions to avoid
No data available

10.5 Incompatible materials
Strong oxidizing agents

10.6 Hazardous decomposition products
In the event of fire: see section 5

SECTION 11: Toxicological information

11.1 Information on toxicological effects

Acute toxicity
LD50 Oral - Rat - > 5,000 mg/kg
Inhalation: No data available
LD50 Dermal - Rabbit - > 2,000 mg/kg

Skin corrosion/irritation
Skin - Rabbit
Result: No skin irritation

Serious eye damage/eye irritation
Eyes - Rabbit
Result: No eye irritation

Respiratory or skin sensitization
No data available

Germ cell mutagenicity
No data available

Carcinogenicity
This product is or contains a component that is not classifiable as to its carcinogenicity based on its IARC, ACGIH, NTP, or EPA classification.

Reproductive toxicity
No data available

Specific target organ toxicity - single exposure

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No data available

Specific target organ toxicity - repeated exposure
No data available

Aspiration hazard
No data available

11.2 Additional Information

RTECS: FI0700000
To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

SECTION 12: Ecological information

12.1 Toxicity
No data available

12.2 Persistence and degradability
No data available

12.3 Bioaccumulative potential
No data available

12.4 Mobility in soil
No data available

12.5 Results of PBT and vPvB assessment
This substance/mixture contains no components considered to be either persistent, bioaccumulative and toxic (PBT), or very persistent and very bioaccumulative (vPvB) at levels of 0.1% or higher.

12.6 Endocrine disrupting properties
No data available

12.7 Other adverse effects
No data available

SECTION 13: Disposal considerations

13.1 Waste treatment methods

Product
Offer surplus and non-recyclable solutions to a licensed disposal company.

Contaminated packaging
Dispose of as unused product.

SECTION 14: Transport information

14.1 UN number
ADR/RID: - IMDG: - IATA: -

14.2 UN proper shipping name
ADR/RID: Not dangerous goods
IMDG: Not dangerous goods
IATA: Not dangerous goods

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14.3 Transport hazard class(es)

ADR/RID: - IMDG: - IATA: -

14.4 Packaging group

ADR/RID: - IMDG: - IATA: -

14.5 Environmental hazards

ADR/RID: no IMDG Marine pollutant: no IATA: no

14.6 Special precautions for user

Further information

Not classified as dangerous in the meaning of transport regulations.

SECTION 15: Regulatory information

15.1 Safety, health and environmental regulations/legislation specific for the substance or mixture

This material safety data sheet complies with the requirements of Regulation (EC) No. 1907/2006.

15.2 Chemical Safety Assessment

For this product a chemical safety assessment was not carried out

SECTION 16: Other information

Further information

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The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Corporation and its Affiliates shall not be held liable for any damage resulting from handling or from contact with the above product. See www.sigma-aldrich.com and/or the reverse side of invoice or packing slip for additional terms and conditions of sale.

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