Toxicological profile for

Methylcyclopentenolone

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

§ IUPAC Systematic name: 2-Hydroxy-3-methylcyclopent-2-en-1-one (CAS RN 80-71-7); 3-methylcyclopentane-1,2-dione (CAS RN 765-70-8) (PubChem)

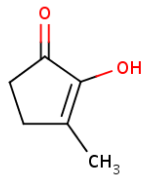
§ Synonyms:

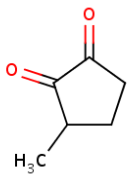
80-71-7: 2-Hydroxy-1-methylcyclopenten-3-one; 2-Hydroxy-3-methyl-2-cyclopenten-1-one; 3-Methyl-2-cyclopentene-2-ol-1-one; 3-Methyl-2-hydroxy-2-cyclopentenone; 3-Methylcyclopent-2-en-2-ol-1-one; 3-Methylcyclopentane-1,2-dione; AI3-36598; BRN 2039308; CCRIS 2940; Corylon; Corylone; Cycloten; Cyclotene; Cyclotene (ordorant); EINECS 201-303-2; EPA Pesticide Chemical Code 004049; Maple lactone; NSC 133445; 2-Hydroxy-3-methylcyclopent-2-enone; 3-Methylcyclopentane-1,2-dione hydrate; UNII-627E92X64B; 2-Cyclopenten-1-one, 2-hydroxy-3-methyl- (ChemIDplus); Flavis No. 07.056, JECFA No. 418 (EFSA, 2015)

765-70-8: 1,2-Cyclopentanedione, 3-methyl-; 3-Methyl-1,2-cyclopentanedione; EINECS 212-154-8; FEMA No. 2700; Methylcyclopentenolone; Methylcyclopentenolone (diketo form); Methylcyclopentenolone (natural); 3-Methylcyclopentane-1,2-dione (ChemIDplus)

§ Gross Formula: $C_6H_8O_2$ (ChemIDplus)

§ Structural Formula (ChemIDplus):





80-71-7 765-70-8

§ Molecular weight (g/mol): 112.13

§ CAS registration number: 80-71-7, 765-70-8

§ Properties:

Melting point: CAS RN 80-71-7: 104-108°C (ChemSpider; EFSA, 2015); 106.5°C (EPISuite; SRC, 2013)

CAS RN 765-70-8: 101-108°C (ChemSpider)

Boiling Point: CAS RN 80-71-7: 178.7°C (ChemSpider)

CAS RN 765-70-8: 178.7±9.0°C (estimated) (ChemSpider); 205.61°C (estimated) (EPISuite)

Solubility in water: CAS RN 80-71-7: 1 g in 72 ml water (EFSA, 2015)

CAS RN 765-70-8: 3.095e+005 mg/l at 25°C (estimated) (EPISuite)

pKa: No data available to us at this time.

Flashpoint: CAS RN 80-71-7: 100.7°C (ChemSpider)

CAS RN 765-70-8: 59.3±4.4°C (estimated) (ChemSpider)

Flammability limits (vol/vol%): No data available to us at this time.

(Auto)ignition temperature: No data available to us at this time.

Decomposition temperature: No data available to us at this time.

Stability: Stable under normal temperatures and pressures.

Vapour pressure: CAS RN 80-71-7: 0.00385 mmHg at 25°C (estimated) (SRC, 2013); 0.0±1.1 mmHg at 25°C (estimated) (ChemSpider); 0.000852 mmHg at 25°C (estimated) (EPISuite)

CAS RN 765-70-8: 1.0±0.3 mmHg at 25°C (estimated) (ChemSpider); 0.396 mmHg at 25°C (estimated) (EPISuite)

log K_{ow}: CAS RN 80-71-7: 0.04; 1.29 (estimated) (EPISuite; SRC, 2013); 0.3 (estimated) (ChemSpider)

CAS RN 765-70-8: 0.035 (ChemSpider)

2. General information

2.1 Exposure

Based on the structural classification of the ingredient as assigned by FDA and JECFA, the estimated maximal exposure to this ingredient via smoking is considered to be below the threshold of toxicological concern as proposed and defined by JECFA (1996) and Munro *et al.*, (1999). Additionally, Ford (2006) indicated correlation between oral and inhalation NOEL (using a database of inhalation NOEL for 350 tobacco ingredients).

Methylcyclopentenolone (CAS RN 80-71-7) is used as a perfuming ingredient in cosmetics in the EU. As taken from CosIng (Cosmetic ingredients database). Available at http://ec.europa.eu/growth/tools-databases/cosing/, accessed July 2017.

Methylcyclopentenolone (CAS RN 765-80-7):

Reported uses (ppm): (FEMA, 1994)

Food Category	Usual	Max.	Food Category	Usual	Max.
Alcoholic beverages	0.93	8.75	Hard candy	16.92	16.92
Baked Goods	12.05	26.40	Meat products	1.95	3.7
Breakfast cereals	40.00	100.00	Nonalcoholic beverages	2.03	5.66
Chewing gum	1.83	7.59	Soft candy	9.35	25.57
Frozen dairy	5.07	16.58	Sweet sauce	8.25	15.75
Gelatins, puddings	4.85	8.68			

Reported individual intake from use as a flavouring: 0.01156 mg/kg bw/day.

As taken from Burdock, 2010.

Both methylcyclopentenolone (CAS RN 80-71-7) and 1,2-cyclopentanedione, 3-methyl- (CAS RN 765-70-8) are listed as fragrance ingredients by the US EPA (2017) and IFRA (2011).

Cyclotene (CAS RN 80-71-7) is registered for use in insect traps (Haz-Map, 2017).

2-Hydroxy-3-methylcyclopent-2-enone (CAS RN 80-71-7) is listed as an ingredient in inside the home products by the US Department of Health and Human Services (2016).

2.2 Combustion products

This ingredient was investigated in a pyrolysis study. Results are given in JTI Study Report(s).

Compound	Two stage heating		One stage heating	
	Abundance	Area%	Abundance	Area%
2-hydroxy-3-methyl-2-cyclopenten-1-one	3608167435	97.30	3617574582	92.73
unknown	47518822	1.28	123204866	3.16
Total ion chromatogram	3709509914	100	3901357378	100

This ingredient was investigated in a pyrolysis study. Results are given in Baker and Bishop (2004) J. Anal. Appl. Pyrolysis, 71, pp. 223-311.

Ingredients CAS Number	Chemical Class	Mol. Wt. (M) Bp or Mp (^{0}C)	Max cig Appln. Level (ppm)	Purity of sample Pyrolysed (%)	Composition of pyrolysate (Compound %)	Max level in smoke (mg)
Methylcyclopentenolone CAS 765-70-8	Alcohol Unsaturated gamma-lactone	M=112 Mp= 105-108	45	99	Methylcyclopentenolone 85.3 Methylcyclohexenone 0.3 3 Unidentified compounds 14.4	19 0.073

In a pyrolysis study, 100% methylcyclopentenolone of added to cigarettes was transferred intact to the smoke (Purkis et al., 2011).

2.3 Ingredient(s) from which it originates

Cyclotene hydrate (CAS RN 80-71-7): natural occurrence in coffee (17-40 mg/kg) (CoE, 2000).

Cyclotene (CAS RN 80-71-7) is reported to be a “product of sugar degradation”.

3-Methyl-1,2-cyclopentanedione (CAS RN 765-70-8) is a "peroxynitrite scavenger isolated from coffee extract".

As taken from Haz-Map, 2017

Maple lactone (CAS RN 80-71-7) is a naturally occurring chemical found in the sap of various trees of the Acer genus and is extracted directly from Acer trees (BPDB, 2015).

3. Status in legislation and other official guidance

Estimated daily per capita intake:

“In the United States, aliphatic acyclic and alicyclic alpha-diketones and alpha-hydroxyketones are generally used as flavouring agents up to average maximum levels of 200 ppm. The total annual volume of the 22 substances in this group is approximately 44 000 kg in Europe (International Organization of the Flavor Industry, 1995) and 56 000 kg in the United States (US National Academy of Sciences, 1970, 1982, 1987). In both Europe and the United States, more

than 95% of the total annual volume is accounted for by three substances: acetoin (No. 405: 19 000 kg/year in Europe and 9200 kg/year in the United States), diacetyl (No. 408: 18 000 kg/year in Europe and 42 000 kg/year in the United States), and methylcyclopentenolone (No. 418: 4700 kg/year in Europe and 3700 kg/year in the United States). Two of these, acetoin and diacetyl, account for more than 90% of the total annual volume in the United States (US National Academy of Sciences, 1987)".

"Nineteen of the 22 aliphatic acyclic and alicyclic alpha-diketones and alpha-hydroxyketones have been identified as natural components of a variety of foods, including fruits, vegetables, cocoa, and coffee (Maarse et al., 1994). Quantitative data have been reported for the natural occurrence of six of these substances, which indicate that the intake as natural components of food is greater than that from their use as flavouring agents (Stofberg & Kirschman, 1985; Stofberg & Grundschober, 1987), with one exception (diacetyl)".

As taken from WHO Food Additives Series 42, available at http://www.inchem.org/documents/jecfa/jecmono/v042je20.htm

JECFA has concluded that the use of methylcyclopentenolone as a food flavour is of "no safety concern", based on current estimated levels of intake from such use (0.89 mg/person/day in Europe and 0.71 mg/person/day in the USA) (JECFA 1999, 2000).

Using the Maximised Survey-derived Daily Intake (MSDI) approach, intake of methyl cyclopentenolone is estimated as 0.57 and 0.71 mg/person/day in the EU and US, respectively.

"No safety concern based on intake calculated by the MSDI approach of the named compound."

As taken from EFSA, 2015.

In an early review of food flavours, the UK Food Additives and Contaminants Committee considered a level of 50 [presumably ppm] to be provisionally acceptable in foodstuffs (MAFF, 1976).

The Council of Europe has classified methylcyclopentenolone in Category A (may be used as a flavouring in foodstuffs). Upper levels of 50 mg/kg in food and 15 mg/kg in beverages were recommended (CoE, 2000).

Methylcyclopentenolone, CAS RN 765-70-8 (Doc No. 0936), is included on the FDA's list of Everything Added to Food in the US (EAFUS) (FDA, 2013), with approval under 21 CFR Section 172.515 (Synthetic flavoring substances and adjuvants) (FDA, 2017).

Both CAS RNs are listed in the US EPA Inert Finder Database (US EPA, 2017) as approved for use in fragrance use pesticide products.

Both CAS RNs are listed in the US EPA TSCA inventory. The TSCA inventory is available at: http://iaspub.epa.gov/sor_internet/registry/substreg/searchandretrieve/searchbylist/search.do

2-Hydroxy-3-methylcyclopent-2-enone (CAS RN 80-71-7) and 3-methylcyclopentane-1,2-dione (CAS RN 765-70-8) are pre-registered under REACH (both "envisaged registration deadline 30 November 2010") (ECHA, 2016a).

Neither 2-hydroxy-3-methylcyclopent-2-enone (CAS RN 80-71-7) nor 3-methylcyclopentane-1,2-dione (CAS RNs 80-71-7 and 765-70-8) are classified for packaging and labelling under Regulation (EC) No. 1272/2008 (ECHA, 2017).

Methyl cyclopentenolone (3-methyl-2-cyclopenten-2-o1-1-one and 2-hydroxy-3-methyl cyclopent-2-en-1-one) appears on the list of "Permitted Additives to Tobacco Products in the United Kingdom" at a maximum level permitted for inclusion in cigarettes/RYO and cigars of 0.005% w/w tobacco and in pipe tobacco of 0.03% (Department of Health, 2003).

3-Methylcyclopentan-1,2-dione (CAS RN 80-71-7) is authorized for use as a flavouring in foodstuffs under Regulation (EU) no. 872.2012 (European Commission, 2012).

Methylcyclopentenolone has been given GRAS (generally recognized as safe) status by FEMA (Hall and Oser, 1965).

Both 2-cyclopenten-1-one, 2-hydroxy-3-methyl- (CAS RN 80-71-7) and 1,2-cyclopentanedione, 3-methyl- (CAS RN 765-70-8) are included on the New Zealand EPA Inventory of Chemicals and the latter may be used as a single component chemical under an appropriate group standard (NZ EPA, 2006). Cylcotene (CAS RN 80-71-7) is also classified by the New Zealand anuthorities (NZ EPA HSNO CCID).

The FEEDAP Panel concluded that 3-methylcyclo-pentan-1,2-dione (07.056; CAS RN 80-71-7) is "safe at the proposed maximum dose level of 5 mg/kg for all target species"

As taken from EFSA, 2016

4. Metabolism/Pharmacokinetics

4.1 Metabolism/metabolites

"Alicyclic diketones are mainly metabolized by reduction to the corresponding diol, followed by glucuronic acid conjugation and excretion."

"In general, alicyclic alpha-diketones are metabolized *via* a reduction pathway (Williams, 1959). In humans, structurally related alicyclic monoketones are reduced to the corresponding alcohols or undergo alpha-hydroxylation and reduction to yield diols, which are excreted as the glucuronic acid conjugates."

"It is anticipated that humans will metabolize low concentrations of aliphatic acyclic methyl ketones principally by oxidation of the terminal methyl group. At higher concentrations, reduction to the diol and subsequent conjugation with glucuronic acid form a competing detoxification pathway. Other alicyclic diketones and hydroxyketones are reduced, conjugated with glucuronic acid, and excreted." As taken from WHO Food Additives Series 42, available at http://www.inchem.org/documents/jecfa/jecmono/v042je20.htm

4.2 Absorption, distribution and excretion

"In rats and mice, orally administered alicyclic diketones are rapidly absorbed from the gastrointestinal tract (Gabriel et al., 1972)."

As taken from WHO Food Additives Series 42, available at http://www.inchem.org/documents/jecfa/jecmono/v042je20.htm

4.3 Interactions

No data available to us at this time.

5. Toxicity

5.1 Single dose toxicity

Species	**Route**	**LD_{50} [mg/kg bw]**
Mouse	Gavage	1350

Rat	Oral	1850
Guinea pig	Gavage	1400

As taken from WHO Food Additives Series 42, available at http://www.inchem.org/documents/jecfa/jecmono/v042je20.htm

Record for 80-71-7:

Species	Route	LDLo [mg/kg bw]	Reference
Rat	Intraperitoneal	500	FCTXAV Food and Cosmetics Toxicology. (London, UK) V.1-19, 1963-81. For publisher information, see FCTOD7. Volume(issue)/page/year: 14,809,1976
Mouse	Intraperitoneal	500	FCTXAV Food and Cosmetics Toxicology. (London, UK) V.1-19, 1963-81. For publisher information, see FCTOD7. Volume(issue)/page/year: 14,809,1976

As taken from RTECS, 1997

Rat oral LD50: >5000 mg/kg bw (CAS RN 80-71-7)

As taken from BPDB, 2015

5.2 Repeated dose toxicity

"Groups of 15 male and 15 female rats aged four to five weeks (strain not specified) were placed on a diet containing 0 or 1% methylcyclo-pentenolone, equivalent to a daily intake of 0 or 500 mg/kg bw, for six months. Each animal was weighed twice weekly and observed frequently for gross appearance and behaviour. The tissues of most animals that died or were killed during the course of the study and of all those kiled at the end of the study were examined grossly. Tissues of representative animals from the control and treated groups (numbers unspecified) were examined microscopically, and the weights of the lungs, heart, liver, kidneys, spleen, and testes were recorded. Haematological parameters were measured in representative animals from the control and treated groups (numbers not specified) at the end of the experiment. There were no statistically significant differences between treated and control animals in any of the parameters measured. The NOEL was 500 mg/kg bw per day (Dow Chemical Co., 1953), which is more than 30 000 the daily *per capita* intake ('eaters only') of 15 and 12 µg/kg bw from its use as a flavouring agent in Europe and the United States, respectively (see Table 2)".

As taken from WHO Food Additives Series 42, available at http://www.inchem.org/documents/jecfa/jecmono/v042je20.htm

5.3 Reproduction toxicity

In a reproductive and developmental toxicity screening study, groups of 10 female Sprague-Dawley rats were administered methylcyclopentenolone, by gavage, at doses of 50, 250 or 500 mg/kg bw/day from 7 days prior to mating, and throughout the mating period and gestation, until postnatal day 4. The maternal no-observed adverse effect level (NOAEL) was <50 mg/kg bw/day (effects on body weight and body weight gain) and the developmental NOAEL was 500 mg/kg bw/day (Volmuth et al. 1990).

Both 2-hydroxy-3-methylcyclopent-2-enone (CAS RN 80-71-7) and 3-methylcyclopentane-1,2-dione (CAS RNs 80-71-7 and 765-70-8) are suspected to be toxic for reproduction. The

CAESAR developmental toxicity model in VEGA (Q)SAR platform predicts that both chemicals are toxicant (good reliability). As taken from ECHA, 2016b.

The reliability and applicability of this QSAR prediction as standalone source of toxicological information is limited and inappropriate for some complex endpoints like reprotoxicity or carcinogenicity. Nevertheless, for the toxicological assessment of this ingredient, this result was still taken into consideration and used within the WoE approach as a supportive tool, in combination with other sources of information when available, like experimental data or appropriate read-across.

5.4 Mutagenicity

Species	Test conditions	Endpoint	Activation	Result	Reference
Rat liver cells	Unscheduled DNA synthesis assay. Tested up to 0.5 mg/plate	DAN damage	Not applicable	-ve	Heck et al 1989, additional data cited in JECFA, 1999
Human leucocytes	Tested at a concentration of 0.35 mM. Examined for chromosome aberrations (abnormal metaphases and chromosome breaks).	Chromosome damage	Without	+ve for abnormal metaphases -ve for chromosome breaks	Withers, 1966
Human lymphocytes	Tested at concentrations of up to 3 mM. Examined for sister chromatid exchanges.	Chromosome effects	Without	+ve (weak)	Jansson et al. 1985 and 1986
Salmonella typhimurium strains TA98, TA100	Ames test. Tested up to 10 mg/plate	Mutation	With and without S9	-ve	Bjeldanes & Chew, 1979
Salmonella typhimurium strains TA98, TA100, TA102	Ames test. Tested up to 22.4 mg/plate	Mutation	With and without S9	-ve	Aeschbacher et al. 1989
Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, TA1538	Ames test. Tested up to 10 mg/plate	Mutation	With and without S9	-ve	Heck et al 1989, additional data cited in JECFA, 1999

“FORMATION OF DIRECT-ACTING GENOTOXIC SUBSTANCES IN NITROSATED SMOKED FISH AND MEAT PRODUCTS: IDENTIFICATION OF SIMPLE PHENOLIC PRECURSORS AND PHENYLDIAZONIUM IONS AS REACTIVE PRODUCTS”.

OHSHIMA H; FRIESEN M; MALAVEILLE C; BROUET I; HAUTEFEUILLE A; BARTSCH H. FOOD CHEM TOXICOL 27:193-203,1989 [EMIC]

GENE-TOX Evaluation B (post-1980); Record for CAS RN 80-71-7:

Species/Cell Type:	Human lymphocytes
Assay Type:	Sister-chromatid exchange (SCE) in vitro
Assay Code:	SCL+D
Results:	Positive
Dose Response:	With dose response
Reference:	EMICBACK/60855; MUTAT RES 169:129-139,1986

Species/Cell Type:	Human
Assay Type:	Sister-chromatid exchange (SCE) in vitro
Assay Code:	SC1+D
Results:	Positive
Dose Response:	With dose response
Reference:	EMICBACK/60855; MUTAT RES 169:129-139,1986

As taken from GENETOX, 1998.

“Methylcyclopentenolone did not induce unscheduled DNA synthesis in rat hepatocytes (Heck et al., 1989)”.

Substance	No	End-point	Test object	Dose	Results	Reference
Methylcyclopentenolone	418	Reverse mutation	S. typhimurium TA1535,TA1537, TA1538, TA98, TA100	10 000 µg/plate	Negative[e]	Heck et al. (1989)
		Unscheduled DNAsynthesis	Rat hepatocytes	500 µg/plate	Negative[a]	Heck et al. (1989)

As taken from WHO Food Additives Series 42, available at http://www.inchem.org/documents/jecfa/jecmono/v042je20.htm

Mutagenicity Studies for CAS RN 80-71-7:

Test System:	AMES SALMONELLA TYPHIMURIUM
Strain Indicator:	TA98
Metabolic Activation:	NONE
Method:	PREINCUBATION
Dose:	0.002-200 UMOL/PLATE (TEST MATERIAL SOLVENT: METHANOL)
Results:	NEGATIVE
Reference:	[AESCHBACHER,HU, WOLLEB,U, LOLIGER,J, SPADONE,JC AND LIARDON,R; CONTRIBUTION OF COFFEE AROMA CONSTITUENTS TO THE MUTAGENICITY OF COFFEE; FOOD CHEM. TOXICOL. 27(4):227-232, 1989]

Test System:	AMES SALMONELLA TYPHIMURIUM
Strain Indicator:	TA100
Metabolic Activation:	NONE
Method:	PREINCUBATION
Dose:	0.002-200 UMOL/PLATE (TEST MATERIAL SOLVENT: METHANOL)
Results:	NEGATIVE
Reference:	[AESCHBACHER,HU, WOLLEB,U, LOLIGER,J, SPADONE,JC AND LIARDON,R; CONTRIBUTION OF COFFEE AROMA CONSTITUENTS TO THE MUTAGENICITY OF COFFEE; FOOD CHEM. TOXICOL. 27(4):227-232, 1989]

Test System:	AMES SALMONELLA TYPHIMURIUM
Strain Indicator:	TA102
Metabolic Activation:	NONE
Method:	PREINCUBATION
Dose:	0.002-200 UMOL/PLATE (TEST MATERIAL SOLVENT: METHANOL)
Results:	NEGATIVE
Reference:	[AESCHBACHER,HU, WOLLEB,U, LOLIGER,J, SPADONE,JC AND LIARDON,R; CONTRIBUTION OF COFFEE AROMA CONSTITUENTS TO THE MUTAGENICITY OF COFFEE; FOOD CHEM. TOXICOL. 27(4):227-232, 1989]

Test System:	AMES SALMONELLA TYPHIMURIUM
Strain Indicator:	TA98
Metabolic Activation:	RAT, LIVER, S-9, AROCLOR 1254
Method:	PREINCUBATION
Dose:	0.002-200 UMOL/PLATE (TEST MATERIAL SOLVENT: METHANOL)
Results:	NEGATIVE
Reference:	[AESCHBACHER,HU, WOLLEB,U, LOLIGER,J, SPADONE,JC AND LIARDON,R; CONTRIBUTION OF COFFEE AROMA CONSTITUENTS TO THE MUTAGENICITY OF COFFEE; FOOD CHEM. TOXICOL. 27(4):227-232, 1989]

Test System:	AMES SALMONELLA TYPHIMURIUM
Strain Indicator:	TA100
Metabolic Activation:	RAT, LIVER, S-9, AROCLOR 1254
Method:	PREINCUBATION
Dose:	0.002-200 UMOL/PLATE (TEST MATERIAL SOLVENT: METHANOL)
Results:	NEGATIVE
Reference:	[AESCHBACHER,HU, WOLLEB,U, LOLIGER,J, SPADONE,JC AND LIARDON,R; CONTRIBUTION OF COFFEE AROMA CONSTITUENTS TO THE MUTAGENICITY OF COFFEE; FOOD CHEM. TOXICOL. 27(4):227-232, 1989]

Test System:	AMES SALMONELLA TYPHIMURIUM
Strain Indicator:	TA102
Metabolic Activation:	RAT, LIVER, S-9, AROCLOR 1254
Method:	PREINCUBATION
Dose:	0.002-200 UMOL/PLATE (TEST MATERIAL SOLVENT: METHANOL)
Results:	NEGATIVE
Reference:	[AESCHBACHER,HU, WOLLEB,U, LOLIGER,J, SPADONE,JC AND LIARDON,R; CONTRIBUTION OF COFFEE AROMA CONSTITUENTS TO THE MUTAGENICITY OF COFFEE; FOOD CHEM. TOXICOL. 27(4):227-232, 1989]

As taken from CCRIS, 1991.

“Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.”

QSAR predictions on mutagenicity:

MultiCASE Ames test: negative.

MultiCase mouse lymphoma test: negative.

Multicase chromosomal aberration test in CHL: netative.

As taken from EFSA, 2015.

3-Methylcyclopentane-1,2-dione (CAS RNs 80-71-7 and 765-70-8) is a suspected mutagen. The Toolbox profiler 'DNA alerts for AMES, MN and CA by OASIS v.1.3' gives an alert for mutagenicity. As taken from ECHA, 2016b.

The reliability and applicability of this QSAR prediction as standalone source of toxicological information is limited and inappropriate for some complex endpoints like reprotoxicity or carcinogenicity. Nevertheless, for the toxicological assessment of this ingredient, this result was still taken into consideration and used within the WoE approach as a supportive tool, in combination with other sources of information when available, like experimental data or appropriate read-across.

5.5 Cytotoxicity

“This study takes the first step towards assessing photo-BCB biocompatibility by evaluating the cytotoxicity and cell adhesion behavior of Cyclotene 4026 coatings exposed to monolayers of glial and fibroblast cells in vitro. It can be concluded from these studies that photo-BCB films deposited on silicon wafers using microfabrication processes did not adversely affect 3T3 fibroblast and T98-G glial cell function in vitro. We also successfully rendered photo-BCB films non-adhesive (no significant fibroblast or glial cell adhesion) with surface immobilized dextran using methods developed for other biomaterials and applications. Future work will further develop prototype photo-BCB microelectrode devices for chronic neural implant applications”. As taken from Ehteshami G et al. 2003. J Biomater Sci Polym Ed. 14(10),1105-16. PubMed, 2010 available at: http://www.ncbi.nlm.nih.gov/pubmed/14661882

5.6 Carcinogenicity

Methylcyclopentenolone was said not to show any tumour promoting activity when tested on mouse skin, but the paper gave no further details (LaVoie *et al*. 1980).

2-Hydroxy-3-methylcyclopent-2-enone (CAS RN 80-71-7) is a suspected carcinogen. The Toolbox profiler carcinogenicity (genotox and nongenotox) alerts by ISS gives an alert for carcinogenicity. As taken from ECHA, 2016b.

The reliability and applicability of this QSAR prediction as standalone source of toxicological information is limited and inappropriate for some complex endpoints like reprotoxicity or carcinogenicity. Nevertheless, for the toxicological assessment of this ingredient, this result was still taken into consideration and used within the WoE approach as a supportive tool, in combination with other sources of information when available, like experimental data or appropriate read-across.

5.7 Irritation / immunotoxicity

There was no evidence of humoral or cell-mediated immunotoxicity in mice given methylcyclopentenolone by gavage in 1% methyl cellulose at dose levels of 125, 250 and 500 mg/kg bw/day(Gaworski *et al*. 1994).

Methylcyclopentenolone was not sensitising or irritating (Moreno 1976; Epstein 1976).

3-Methylcyclopentane-1,2-dione (CAS RNs 80-71-7 and 765-70-8) is a suspected skin sensitizer. The Toolbox profiler 'Protein binding alerts for skin sensitization by OASIS v1.3' gives an alert for skin sensitization. The CAESAR skin sensitization model in VEGA (Q)SAR platform predicts that the chemical is a sensitizer (moderate reliability). As taken from ECHA, 2016b.

The reliability and applicability of this QSAR prediction as standalone source of toxicological information is limited and inappropriate for some complex endpoints like reprotoxicity or carcinogenicity. Nevertheless, for the toxicological assessment of this ingredient, this result was still taken into consideration and used within the WoE approach as a supportive tool, in combination with other sources of information when available, like experimental data or appropriate read-across.

5.8 All other relevant types of toxicity

Total particulate matter (TPM) from heated (tobacco or nicotine) product(s) containing Methylcyclopentenolone (765-70-8; 80-71-7) or Cyclotene (765-70-8) was tested in a battery of *in vitro* and/or *in vivo* test(s). Within the sensitivity and specificity of the bioassay(s) the activity of the TPM was not increased by the addition of Methylcyclopentenolone (765-70-8; 80-71-7) or Cyclotene (765-70-8) when compared to TPM from 3R4F cigarettes. The table below provides tested level(s) and specific endpoint(s).

Endpoint	**Tested level (ppm)**	**Reference**
In vitro genotoxicity	26 (Cyclotene) 19.5 (Methylcyclopentenolone)	JTI KB Study Report(s)
In vitro cytotoxicity	26 (Cyclotene) 19.5 (Methylcyclopentenolone)	JTI KB Study Report(s)

6. Functional effects on

6.1 Broncho/pulmonary system

No data available to us at this time.

6.2 Cardiovascular system

Methylcyclopentenolone injected intraperitoneally into mice and rats in daily doses of 100 mg/kg bw for 20 days had no effect on the number or activity of leucocytes (Shugaev 1959).

6.3 Nervous system

No data available to us at this time.

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

“Prooxidant properties of furanone compounds including 2,5-furanone (furaneol, 4-hydroxy-2,5-dimethyl-furan-3-one), 4,5-furanone (4,5-dimethyl-3-hydroxy-2(5H)-furanone) (sotolone) and cyclotene (2-hydroxy-3-methyl-2-cyclopenten-1-one) were analyzed in relation to the metal-reducing activity. Only 2.5-furanone known as a "strawberry or pineapple furanone" inactivated aconitase the most sensitive enzyme to active oxygen in the presence of ferrous sulfate, suggesting the furaneol/iron-mediated generation of reactive oxygen species. 2,5-Furanone caused strand scission of pBR322 DNA in the presence of copper. Treatment of calf thymus DNA with 2,5-furanone plus copper produced 8-hydroxy-2'-deoxyguanosine in DNA. 2,5-Furanone showed a potent copper-reducing activity, and thus, DNA strand breaks and the formation of 8-hydroxy-2'-deoxyguanosine by 2,5-furanone can be initiated by the production of superoxide radical through the reduction of cupric ion to cuprous ion, resulting in the conversion to hydrogen peroxide and hydroxyl radical. However, an isomer and analog of 2,5-furanone, 4,5-furanone and cyclotene, respectively, did not show an inactivation of aconitase, DNA injuries including strand breakage and the formation of 8-hydroxy-2'-deoxyguanosine, and copper-reducing activity. Cytotoxic effect of 2,5-furanone with hydroxyketone structure can be explained by its prooxidant properties: furaneol/transition metal complex generates reactive oxygen species causing the inactivation of aconitase and the formation of DNA base damage by hydroxyl radical.” As taken from Murakami K et al. 2007. Food Chem Toxicol. 45(7), 1258-62. PubMed, 2010 available at: http://www.ncbi.nlm.nih.gov/pubmed/17316945

“It has been known that reactive oxygen and nitrogen species such as nitric oxide (NO), superoxide radical (*O2-) and their byproduct peroxynitrite (ONOO-) induce cellular and tissue injury, ultimately resulting in several human diseases. In this study, we examined scavenging effects of 3-methyl-1,2-cyclopentanedione (MCP) from coffee extract on the reactivity of those toxic molecules. MCP significantly inhibited both the oxidation of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) by reactive oxygen species (ROS) (mainly *O2-) from kidney homogenate (41% at 100 microM) and the generation of fluorescent 4,5-diaminofluorescein (DAF-2) by NO from sodium nitroprusside (IC50 (concn producing 50% inhibition), 63.8 microM). More potently, however, MCP suppressed the oxidation of dihydrorhodamine 123 (DHR 123) to fluorescent rhodamine 123 mediated by authentic ONOO- with an IC50 value of 3.3 microM. The neutralizing effect of the reactivity of ONOO- by MCP was due to electron donation, not nitration of the compound. Additionally, MCP also decreased ONOO- formation of nitrotyrosine adducts of glutathione (GSH) reductase, and consequently protected the enzyme activity of GSH reductase against decreasing by ONOO-, indicating that MCP may prevent ONOO- -induced

damage of GSH reductase. Furthermore, MCP only weakly suppressed NO production, which is one of the upstream sources of ONOO- in-vivo, suggesting that NO production may be not a pharmacological target for MCP. Taken together, our results suggest that MCP may be regarded as a selective regulator of ONOO- -mediated diseases via direct scavenging activity of ONOO". As taken from Kim AR et al. 2002. J Pharm Pharmacol. 54(10), 1385-92. PubMed, 2010 available at: http://www.ncbi.nlm.nih.gov/pubmed/12396301

7. Addiction

JTI is not aware of any information that demonstrates that this ingredient has any addictive effect.

8. Burnt ingredient toxicity

This ingredient was considered as part of an overall safety assessment of ingredients added to tobacco in the manufacture of cigarettes. An expert panel of toxicologists reviewed the open literature and internal toxicology data of 5 tobacco companies to evaluate a composite list of ingredients used in the manufacture of cigarettes. The conclusion of this report was that these ingredients did not increase the inherent biological activity of tobacco cigarettes, and are considered to be acceptable under conditions of intended use (Doull et al., 1994 & 1998).

Tobacco smoke condensates from cigarettes containing methylcyclopentenolone and an additive free, reference cigarettes were tested in a battery of in vitro and/or in vivo test(s). Within the sensitivity and specificity of the bioassay(s) the activity of the condensate was not changed by the addition of methylcyclopentenolone. Table below provides tested level(s) and specific endpoint(s).

Endpoint	Tested level (ppm)	Reference
Smoke chemistry	124 (80-71-7)	Carmines, 2002 & Rustemeier et al., 2002
	53 (765-70-8)	Baker et al., 2004a
	26 (765-70-8) 65 (765-70-8) 220 (Cigar)	JTI KB Study Report(s)
	2,680	Gaworski et al., 2011 & Coggins et al., 2011f
	108 (80-71-7) 18 (765-70-8)	Roemer et al., 2014
In vitro genotoxicity	124 (80-71-7)	Carmines, 2002 & Roemer et al., 2002
	53 (765-70-8)	Baker et al., 2004c
	26 (80-71-7)	Renne et al., 2006
	26 (765-70-8) 220 (Cigar)	JTI KB Study Report(s)
	279 (765-70-8)	fGLH Study Report (2010)
	2,680	Gaworski et al., 2011 & Coggins et al., 2011f
	108 (80-71-7) 18 (765-70-8)	Roemer et al., 2014
In vitro cytotoxicity	124 (80-71-7)	Carmines, 2002 & Roemer et al., 2002
	53 (765-70-8)	Baker et al., 2004c

	220 (Cigar)	JTI KB Study Report(s)
	279 (765-70-8)	fGLH Study Report (2010)
	2,680	Gaworski et al., 2011 & Coggins et al., 2011f
	108 (80-71-7) 18 (765-70-8)	Roemer et al., 2014
Inhalation study	23 (80-71-7)	Gaworski et al., 1998
	124 (80-71-7)	Carmines, 2002 & Vanscheeuwijck et al., 2002
	53 (765-70-8)	Baker et al., 2004c
	26 (80-71-7)	Renne et al., 2006
	26 (765-70-8)	JTI KB Study Report(s)
	108 (80-71-7) 18 (765-70-8)	Schramke et al., 2014
Skin painting	23 (80-71-7)	Gaworski et al., 1999
	26 (765-70-8)	JTI KB Study Report(s)
In vivo genotoxicity	108 (80-71-7) 18 (765-70-8)	Schramke et al., 2014
	220 (cigar)	JTI KB Study Report(s)

Transfer to the smoke

Methylcyclopentenolone was added to cigarettes and the amount that transferred to smoke unchanged was measured at 5.58 ±0.57% (McDermott 1983).

In a pyrolysis study, 100% methylcyclopentenolone of added to cigarettes was transferred intact to the smoke (Purkis et al., 2011).

“Cigarette smoke condensate is known to enhance the frequency of sister-chromatid exchanges (SCE) in human lymphocytes in vitro and some of the activity has been found in the most volatile part of the particulate phase, the semivolatile fraction. In this study we have investigated the chemical composition and the SCE-inducing activity of the weakly acidic, semivolatile fraction of a cigarette smoke condensate. A number of individual weakly acidic compounds were also tested for their SCE-inducing effects. The weakly acidic fraction was separated by preparative gel chromatography into 11 subfractions (F1-F11). The chemical composition was determined by gas chromatography and gas chromatography-mass spectrometry. Measurements of the effects on SCE in human lymphocytes were used to evaluate the genotoxic effects. All fractions except F11 induced SCE in a dose-dependent way. The most active fraction was F4 which contained mainly alkyl-2-hydroxy-2-cyclopenten-1-ones. The individual compounds to be tested for induction of SCE were selected on the basis of their abundance in the weakly acidic subfractions and on the basis of their occurrence in the environment. Of 23 tested compounds, most of which were alkylphenols, 7 induced SCE, i.e., catechol, 2-(1-propenyl)phenol, cyclotene, maltol, isoeugenol, 2-methoxyphenol (guaiacol) and vanillin. Many of these are important flavor components that occur not only in tobacco and tobacco smoke but also in food, candies, beverages and perfumes”. As taken from Jansson T et al. 1986. Mutat Res. 169(3),129-39. PubMed, 2010 available at: http://www.ncbi.nlm.nih.gov/pubmed/3951466

“Water-soluble portion of the smoke condensate of cellulose cigarette was studied and 42 compounds-10 lactones, 6 cyclopentanones (or cyclopentenones), 5 pyrans and other

compounds-were either positively or tentatively identified. Of these 42 compounds, 12, such as succinaldehyde, 5-hydroxy-2-pentenone, etc., were identified for the 1st time in cellulose pyrolyzate. Semi-quantitative estimation of some of the major components in water-soluble portion revealed that 1,6-anhydroglucopyranose (levoglucosan) was the most abundant, followed by 2-hydroxy-3-methyl-2-cyclopentenone (cyclotene), acetol, 2-hydroxy-5-pentanolide, etc. Glycolaldehyde which seems to be more abundant than cyclotene on chromatogram could not be estimated because of peak tailing. Of these water-soluble components, succinaldehyde has a very pungent and irritating effect and seems to be a cause of the pungent and irritating odor of cellulose cigarette smoke". As taken from Sakuma H and Sugawara S. 1979. Agric Biol Chem. 43(7), 1585-1590.

9. Ecotoxicity

9.1 Environmental fate

The Ecological Categorization Results from the Canadian Domestic Substances List state that neither 2-cyclopenten-1-one, 2-hydroxy-3-methyl- (CAS RN 80-71-7) nor 1,2-cyclopentanedione, 3-methyl- (CAS RN 765-70-8) are persistent in the environment:

	CAS RN 80-71-7	CAS RN 765-70-8
Media of concern leading to Categorization	Water	Water
Experimental Biodegradation half-life (days)	Not Available	Not Available
Predicted Ultimate degradation half-life (days)	15	15
MITI probability of biodegradation	0.8346	0.4138
TOPKAT probability of biodegradation	1	1
EPI Predicted Ozone reaction half-life (days)	0.155	999
EPI Predicted Atmospheric Oxidation half-life (days)	0.1047	0.9449

Data accessed July 2017 on the OECD website: http://webnet.oecd.org/CCRWeb/Search.aspx

EPISuite provides the following information for CAS RN 80-71-7:

Henrys Law Constant (25 deg C) [HENRYWIN v3.20]:

Bond Method :	3.50E-005 atm-m3/mole (3.55E+000 Pa-m3/mole)
Group Method:	Incomplete
Henrys LC [via VP/WSol estimate using User-Entered or Estimated values]:	HLC: 1.479E-008 atm-m3/mole (1.498E-003 Pa-m3/mole) VP: 0.000852 mm Hg (source: MPBPVP) WS: 8.5E+003 mg/L (source: WSKOWWIN)

Log Octanol-Air Partition Coefficient (25 deg C) [KOAWIN v1.10]:

Log Kow used:	1.29 (KowWin est)
Log Kaw used:	-2.844 (HenryWin est)
Log Koa (KOAWIN v1.10 estimate):	4.134
Log Koa (experimental database):	None

Probability of Rapid Biodegradation (BIOWIN v4.10):

Biowin1 (Linear Model): Biowin2 (Non-Linear Model) : Biowin3 (Ultimate Survey Model): Biowin4 (Primary Survey Model) :	0.8597 0.8891 3.0889 (weeks) 3.7932 (days)

Biowin5 (MITI Linear Model) : Biowin6 (MITI Non-Linear Model): Biowin7 (Anaerobic Linear Model):	0.6973 0.8346 0.2574
Ready Biodegradability Prediction:	YES

Hydrocarbon Biodegradation (BioHCwin v1.01):

Structure incompatible with current estimation method!

Sorption to aerosols (25 Dec C)[AEROWIN v1.00]:

Vapor pressure (liquid/subcooled):	0.715 Pa (0.00536 mm Hg)
Log Koa (Koawin est):	4.134
Kp (particle/gas partition coef. (m3/ug)): Mackay model: Octanol/air (Koa) model:	 4.2E-006 3.34E-009

Fraction sorbed to airborne particulates (phi):

Junge-Pankow model:	0.000152
Mackay model:	0.000336
Octanol/air (Koa) model:	2.67E-007

Atmospheric Oxidation (25 deg C) [AopWin v1.92]:

Hydroxyl Radicals Reaction:

OVERALL OH Rate Constant =	102.1587 E-12 cm3/molecule-sec
Half-Life =	0.105 Days (12-hr day; 1.5E6 OH/cm3)
Half-Life =	1.256 Hrs

Ozone Reaction:

OVERALL Ozone Rate Constant =	7.393750 E-17 cm3/molecule-sec
Half-Life =	0.155 Days (at 7E11 mol/cm3)
Half-Life =	3.720 Hrs
Fraction sorbed to airborne particulates (phi): 0.000244 (Junge-Pankow, Mackay avg) 2.67E-007 (Koa method) Note: the sorbed fraction may be resistant to atmospheric oxidation	

Soil Adsorption Coefficient (KOCWIN v2.00):

Koc :	1.229 L/kg (MCI method)
Log Koc:	0.090 (MCI method)
Koc :	26.47 L/kg (Kow method)
Log Koc:	1.423 (Kow method)

Aqueous Base/Acid-Catalyzed Hydrolysis (25 deg C) [HYDROWIN v2.00]:

Rate constants can NOT be estimated for this structure!

Volatilization from Water:

Henry LC: 3.5E-005 atm-m3/mole (estimated by Bond SAR Method)

Half-Life from Model River:	18.79 hours
Half-Life from Model Lake:	293.8 hours (12.24 days)

Removal In Wastewater Treatment:

Total removal:	3.77 percent
Total biodegradation:	0.09 percent
Total sludge adsorption:	1.80 percent
Total to Air:	1.87 percent

(using 10000 hr Bio P,A,S)

Level III Fugacity Model:

	Mass Amount (percent)	Half-Life (hr)	Emissions (kg/hr)
Air	0.344	1.5	1000
Water	48.4	360	1000
Soil	51.2	720	1000
Sediment	0.091	3.24e+003	0

Persistence Time: 283 hr

EPISuite provides the following information for CAS RN 765-70-8:

Henrys Law Constant (25 deg C) [HENRYWIN v3.20]:

Bond Method :	1.53E-007 atm-m3/mole (1.55E-002 Pa-m3/mole)
Group Method:	Incomplete
Henrys LC [via VP/WSol estimate using User-Entered or Estimated values]:	HLC: 1.888E-007 atm-m3/mole (1.913E-002 Pa-m3/mole) VP: 0.396 mm Hg (source: MPBPVP) WS: 3.1E+005 mg/L (source: WSKOWWIN)

Log Octanol-Air Partition Coefficient (25 deg C) [KOAWIN v1.10]:

Log Kow used:	-0.54 (KowWin est)
Log Kaw used:	-5.204 (HenryWin est)
Log Koa (KOAWIN v1.10 estimate):	4.664
Log Koa (experimental database):	None

Probability of Rapid Biodegradation (BIOWIN v4.10):

Biowin1 (Linear Model):	0.6942
Biowin2 (Non-Linear Model) :	0.8048
Biowin3 (Ultimate Survey Model):	2.9514 (weeks)
Biowin4 (Primary Survey Model) :	3.6860 (days-weeks)
Biowin5 (MITI Linear Model) :	0.4309
Biowin6 (MITI Non-Linear Model):	0.4138
Biowin7 (Anaerobic Linear Model):	0.5559
Ready Biodegradability Prediction:	NO

Hydrocarbon Biodegradation (BioHCwin v1.01):

Structure incompatible with current estimation method!

Sorption to aerosols (25 Dec C)[AEROWIN v1.00]:

Vapor pressure (liquid/subcooled):	48.7 Pa (0.365 mm Hg)

Log Koa (Koawin est):	4.664
Kp (particle/gas partition coef. (m3/ug)): Mackay model: Octanol/air (Koa) model:	 6.16E-008 1.13E-008

Fraction sorbed to airborne particulates (phi):

Junge-Pankow model:	2.23E-006
Mackay model:	4.93E-006
Octanol/air (Koa) model:	9.06E-007

Atmospheric Oxidation (25 deg C) [AopWin v1.92]:

Hydroxyl Radicals Reaction:

OVERALL OH Rate Constant =	11.3191 E-12 cm3/molecule-sec
Half-Life =	0.945 Days (12-hr day; 1.5E6 OH/cm3)
Half-Life =	11.339 Hrs
Ozone Reaction:	No Ozone Reaction Estimation
Fraction sorbed to airborne particulates (phi):	3.58E-006 (Junge-Pankow, Mackay avg) 9.06E-007 (Koa method)
Note: the sorbed fraction may be resistant to atmospheric oxidation	

Soil Adsorption Coefficient (KOCWIN v2.00):

Koc :	1 L/kg (MCI method)
Log Koc:	0.000 (MCI method)
Koc :	1.472 L/kg (Kow method)
Log Koc:	0.168 (Kow method)

Aqueous Base/Acid-Catalyzed Hydrolysis (25 deg C) [HYDROWIN v2.00]:

Rate constants can NOT be estimated for this structure!

Volatilization from Water:

Henry LC: 1.53E-007 atm-m3/mole (estimated by Bond SAR Method)

Half-Life from Model River:	4053 hours (168.9 days)
Half-Life from Model Lake:	4.431E+004 hours (1846 days)

Removal In Wastewater Treatment:

Total removal:	1.86 percent
Total biodegradation:	0.09 percent
Total sludge adsorption:	1.76 percent
Total to Air:	0.01 percent

(using 10000 hr Bio P,A,S)

Level III Fugacity Model:

	Mass Amount (percent)	Half-Life (hr)	Emissions (kg/hr)
Air	1.49	22.7	1000
Water	42.9	360	1000

Soil	55.5	720	1000
Sediment	0.0804	3.24e+003	0

Persistence Time: 418 hr

9.2 Aquatic toxicity

The Ecological Categorization Results from the Canadian Domestic Substances List state that neither 2-cyclopenten-1-one, 2-hydroxy-3-methyl- (CAS RN 80-71-7) nor 1,2-cyclopentanedione, 3-methyl- (CAS RN 765-70-8) are inherently toxic to aquatic organisms.

	CAS RN 80-71-7	CAS RN 765-70-8
Pivotal value for iT (mg/l)	209.329	2066.47168
Toxicity to fish (LC50 in mg/l) as predicted by Ecosar v0.99g	209.329	20,291.752
Toxicity to fish (LC50 in mg/l) as predicted by Oasis Forecast M v1.10	751.3243	-
Toxicity to fish (LC50 in mg/l) as predicted by Aster	2,555.79525	43,659.508
Toxicity to fish (LC50 in mg/l) as predicted by PNN	1,347.958	2,066.47168
Toxicity to daphnia (EC50 in mg/l) as predicted by Topkat v6.1	-	0.7828
Toxicity to fish, daphnia, algae or mysid shrimp (EC50 or LC50 in mg/l) as predicted by Ecosar v0.99g	625.359	9,854.157
Chronic toxicity to daphnia or algae (EC50 in mg/l) as predicted by Ecosar v0.99g	-	307.978
Toxicity to fish (LC50 in mg/l) as predicted by Neutral Organics QSAR in Ecosar v0.99g	3.86E+000	2.03E+004

Data accessed July 2017 on the OECD website: http://webnet.oecd.org/CCRWeb/Search.aspx

ECOSAR Version 1.11 provides the following aquatic toxicity data for CAS RN 80-71-7:

Values used to Generate ECOSAR Profile
Log Kow: 1.288 (EPISuite Kowwin v1.68 Estimate)
Wat Sol: 2537 (mg/L, EPISuite WSKowwin v1.43 Estimate)

ECOSAR v1.11 Class-specific Estimations
Vinyl/Allyl Ketones
Vinyl/Allyl Alcohols

ECOSAR Class	Organism	Duration	End Pt	Predicted mg/L (ppm)
Vinyl/Allyl Ketones :	Fish	96-hr	LC50	398.321
Vinyl/Allyl Ketones :	Daphnid	48-hr	LC50	330.671
Vinyl/Allyl Ketones :	Green Algae	96-hr	EC50	169.269
Vinyl/Allyl Ketones :	Fish		ChV	161.213
Vinyl/Allyl Ketones :	Daphnid		ChV	43.466 !
Vinyl/Allyl Ketones :	Green Algae		ChV	40.355 !
Vinyl/Allyl Ketones :	Fish (SW)	96-hr	LC50	10572.408 *
Vinyl/Allyl Ketones :	Mysid (SW)	96-hr	LC50	330.853
Vinyl/Allyl Ketones :	Fish (SW)		ChV	1192.620
Vinyl/Allyl Ketones :	Mysid (SW)		ChV	59.735 !
Vinyl/Allyl Alcohols :	Fish	96-hr	LC50	2.775

Vinyl/Allyl Alcohols :	Daphnid	48-hr	LC50	0.364
Vinyl/Allyl Alcohols :	Green Algae	96-hr	EC50	43.415
Vinyl/Allyl Alcohols :	Fish		ChV	0.106 !
Vinyl/Allyl Alcohols :	Daphnid		ChV	0.022 !
Vinyl/Allyl Alcohols :	Green Algae		ChV	6.027

Neutral Organic SAR :	Fish	96-hr	LC50	401.575
(Baseline Toxicity) :	Daphnid	48-hr	LC50	213.509
	Green Algae	96-hr	EC50	121.190
	Fish		ChV	36.324
	Daphnid		ChV	17.340
	Green Algae		ChV	27.415

Note: * = asterisk designates: Chemical may not be soluble enough to measure this predicted effect. If the effect level exceeds the water solubility by 10X, typically no effects at saturation (NES) are reported.

NOTE: ! = exclamation designates: The toxicity value was estimated through application of acute-to-chronic ratios per methods outlined in the ECOSAR Methodology Document provided in the ECOSAR Help Menu.

ECOSAR Version 1.11 provides the following aquatic toxicity data for CAS RN 765-70-8:

Values used to Generate ECOSAR Profile
Log Kow: -0.540 (EPISuite Kowwin v1.68 Estimate)
Wat Sol: 3.095E+005 (mg/L, EPISuite WSKowwin v1.43 Estimate)

ECOSAR v1.11 Class-specific Estimations
Not Related to an Existing ECOSAR Class
Estimates provided below use the Neutral Organics QSAR equations represent baseline toxicity potential (minimum toxicity) assuming a simple non-polar narcosis model. Without empirical data on structurally similar chemicals, it is uncertain if this substance will present significantly higher toxicity above baseline estimates.

ECOSAR Class	**Organism**	**Duration**	**End Pt**	**Predicted mg/L (ppm)**
Neutral Organics :	Fish	96-hr	LC50	17602.865
Neutral Organics :	Daphnid	48-hr	LC50	7905.425
Neutral Organics :	Green Algae	96-hr	EC50	2232.938
Neutral Organics :	Fish		ChV	1304.778
Neutral Organics :	Daphnid		ChV	401.375
Neutral Organics :	Green Algae		ChV	346.853
Neutral Organics :	Fish (SW)	96-hr	LC50	21826.248
Neutral Organics :	Mysid	96-hr	LC50	90651.805
Neutral Organics :	Fish (SW)		ChV	502.278
Neutral Organics :	Mysid (SW)		ChV	16633.643

Fish 96-hour LC50 >100 mg/l (CAS RN 80-71-7)

Aquatic invertebrates 48-hour EC50 >100 mg/l (CAS RN 80-71-7)

As taken from BPDB, 2015

9.3 Sediment toxicity

No data available to us at this time.

9.4 Terrestrial toxicity

ECOSAR Version 1.11 provides the following terrestrial toxicity data for CAS RN 765-70-8:

Values used to Generate ECOSAR Profile
Log Kow: -0.540 (EPISuite Kowwin v1.68 Estimate)
Wat Sol: 3.095E+005 (mg/L, EPISuite WSKowwin v1.43 Estimate)

ECOSAR v1.11 Class-specific Estimations
Not Related to an Existing ECOSAR Class
Estimates provided below use the Neutral Organics QSAR equations represent baseline toxicity potential (minimum toxicity) assuming a simple non-polar narcosis model. Without empirical data on structurally similar chemicals, it is uncertain if this substance will present significantly higher toxicity above baseline estimates.

ECOSAR Class	**Organism**	**Duration**	**End Pt**	**Predicted mg/L (ppm)**
Neutral Organics :	Earthworm	14-day	LC50	357.543

Bird acute LD50 >2000 mg/kg (CAS RN 80-71-7)

Honeybee 48-hour LD50 >100 µg/bee (CAS RN 80-71-7)

Earthworm 14-day LC50 >1000 mg/kg (CAS RN 80-71-7)

As taken from BPDB, 2015

Predicted environmental concentration (PEC) values (calculated for lamb manure)

EU register name	CAS no.	Dose mg/kg	PEC_{soil} (µg/kg)	$PEC_{porewater}$ (µg/L)	$PEC_{surfacewater}$ (µg/L)
3-Methylcyclopentan-1,2-dione	80-71-7	5	107	765	255

As taken from EFSA, 2016

9.5 All other relevant types of ecotoxicity

The Ecological Categorization Results from the Canadian Domestic Substances List state that neither 2-cyclopenten-1-one, 2-hydroxy-3-methyl- (CAS RN 80-71-7) nor 1,2-cyclopentanedione, 3-methyl- (CAS RN 765-70-8) is bioaccumulative in the environment.

	CAS RN 80-71-7	CAS RN 765-70-8
Log Kow predicted by KowWin	1.29	-0.54
Log BAF T2MTL predicted by Gobas	0.336113190481144	0.0000540371955876
Log BCF 5% T2LTL predicted by Gobas	0.288552744151573	0.0005144646215092
Log BCF Max predicted by OASIS	1.36114386877595	0.99832835101101
Log BCF predicted by BCFWIN	0.292	0.5

Data accessed July 2017 on the OECD website: http://webnet.oecd.org/CCRWeb/Search.aspx

EPISuite provides the following information for CAS RN 80-71-7:

Bioaccumulation Estimates (BCFBAF v3.01):

Log BCF from regression-based method:	0.517 (BCF = 3.287 L/kg wet-wt)
Log Biotransformation Half-life (HL):	-1.2328 days (HL = 0.05851 days)
Log BCF Arnot-Gobas method (upper trophic):	0.356 (BCF = 2.268)
Log BAF Arnot-Gobas method (upper trophic):	0.356 (BAF = 2.268)
log Kow used:	1.29 (estimated)

EPISuite provides the following information for CAS RN 765-70-8:

Bioaccumulation Estimates (BCFBAF v3.01):

Log BCF from regression-based method:	0.500 (BCF = 3.162 L/kg wet-wt)
Log Biotransformation Half-life (HL):	-1.5404 days (HL = 0.02881 days)
Log BCF Arnot-Gobas method (upper trophic):	-0.041 (BCF = 0.9095)
Log BAF Arnot-Gobas method (upper trophic):	-0.041 (BAF = 0.9095)
log Kow used:	-0.54 (estimated)

10. References

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11. Other information

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SCIENTIFIC OPINION

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Scientific Opinion on Flavouring Group Evaluation 213, Revision 2 (FGE.213Rev2): Consideration of genotoxic potential for α,β-unsaturated alicyclic ketones and precursors from chemical subgroup 2.7 of FGE.19

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)

Abstract

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) of the European Food Safety Authority (EFSA) was requested to evaluate the genotoxic potential of 26 flavouring substances from subgroup 2.7 of FGE.19 in Flavouring Group Evaluation (FGE) 213. In the first version of FGE.213 the Panel concluded, based on available genotoxicity data, that a concern regarding genotoxicity could be ruled out for 11 substances [FL-nos: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168], but for the remaining 15 substances in subgroup 2.7 further genotoxicity data were required. Based on new submitted genotoxicity data, the Panel concluded in FGE.213Rev1 that the concern regarding genotoxicity could be ruled out for 13 substances in subgroup 2.7 [FL-nos: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] but not for maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525]. In FGE.213Rev2, new data on maltol were considered and the Panel concluded that for maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525] in food the concern for genotoxicity could be ruled out. Moreover, the Panel reconsidered the available data on *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127], based on new data on the structurally related substance pulegone, and concluded that additional genotoxicity data are needed to rule out the concern for genotoxicity of *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127].

Keywords: FGE.213, α,β-unsaturated alicyclic ketones, flavouring substances, safety evaluation, subgroup 2.7, FGE.19

Requestor: European Commission

Question numbers: EFSA-Q-2015-00138, EFSA-Q-2015-00139

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Summary

Following a request from the European Commission, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) was asked to deliver a scientific opinion on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Scientific Panel was asked to evaluate flavouring substances using the procedure referred to in Commission Regulation EC No 1565/2000 (hereafter 'the Procedure').

The Flavouring Group Evaluation (FGE) 213 concerns 26 substances, corresponding to subgroup 2.7 of FGE.19. Twenty-three of the substances are α,β-unsaturated alicyclic ketones [Flavour Information System (FL)-nos: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] and three are precursors for such ketones [FL-nos: 02.106, 09.305 and 09.525].

In the first version of FGE.213 the Panel concluded that the genotoxicity concern for ethyl maltol [FL-no: 07.047], 3-ethylcyclopentan-1,2-dione [FL-no: 07.057] and the nine structurally related substances [FL-nos: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080] could be ruled out and the 11 substances could accordingly be evaluated through the Procedure.

For maltol [FL-no: 07.014], a micronucleus assay after oral application was required in addition to an *in vivo* comet assay in order to clarify the genotoxic potential. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

The remaining 13 substances (including two precursors of a ketone) [FL-nos: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] could not be evaluated through the Procedure. Accordingly, additional data on genotoxicity were required for representatives of these 13 substances.

The Flavour Industry informed that it no longer supports the representative flavouring substance, piperitenone oxide [FL-no: 16.044], for which the Panel requested additional data. In FGE.213Rev1, one additional substance has been included in subgroup 2.7, *tr*-1-(2,6,6-trimethyl-1-cyclohexen-1-yl)but-2-en-1-one [FL-no: 07.224], which is structurally related to the other substances for which the genotoxic potential could not be ruled out.

In FGE.213Rev1, the Panel evaluated the new data submitted by the Flavour Industry in response to the data request presented in FGE.213. Based on these new data, the Panel concluded that the genotoxicity concern could be ruled out for the representative substances β-ionone [FL-no: 07.008], β-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089], 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] and the nine substances that they represent [FL-nos: 02.106, 07.010, 07.041, 07.108, 07.127, 07.136, 07.200, 07.224 and 09.305].

In the case of maltol, positive results were observed in an *in vitro* micronucleus assay in human peripheral blood lymphocytes and in an *in vivo* micronucleus assay in mouse bone marrow after intraperitoneal application. Maltol was also tested in rats (administered by gavage) in a combined bone marrow micronucleus assay and comet assay in liver. Both tests showed negative results, but no clinical signs and no bone marrow toxicity were observed. To investigate the systemic exposure, plasma bioanalysis was performed, but results were inconsistent. Owing to the intended use of maltol as a food-flavouring agent, the *in vivo* study performed with administration of maltol by gavage is considered more relevant than the study performed by intraperitoneal application. Therefore, the Panel concluded in Revision 1 of this FGE that for maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525] the concern for genotoxicity could not be ruled out.

The Flavour Industry has submitted a new plasma bioanalysis for maltol, which is evaluated in the present revision of FGE.213 (FGE.213Rev2). The Panel considered this new plasma bioanalysis and concluded that it seems justifiable to assume that animals were systemically exposed to maltol and that the bone marrow was exposed in the *in vivo* micronucleus assay. Therefore, the negative result of the *in vivo* micronucleus assay can be considered reliable and, accordingly, the concern for genotoxicity for maltol [FL-no: 07.014] and for maltyl isobutyrate [FL-no: 09.525] in food is ruled out; both substances were evaluated by JECFA before 2000 and no EFSA consideration is required.

Moreover, the Panel reconsidered the available data on *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127], based on new data on the structurally related substance pulegone and concluded that additional genotoxicity data are needed to rule out the concern for genotoxicity on *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127].

Table of contents

1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

The use of flavourings is regulated under Regulation (EC) No 1334/2008[1] of the European Parliament and Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of Article 9(a) of this Regulation, an evaluation and approval are required for flavouring substances.

The Union List of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012[2]. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) N° 1565/2000.

On 10 April 2014 the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids adopted an opinion on Flavouring Group Evaluation 213, Revision 1 (FGE.213Rev1): Consideration of genotoxic potential for α,β-unsaturated alicyclic ketones and precursors from chemical subgroup 2.7 of FGE.19.[3]

The Panel concluded that, for maltol [FL-no: 07.014] and for maltyl isobutyrate [FL-no: 09.525] the Panel's concern with respect to genotoxicity could not be ruled out and subsequently additional data are requested. In particular it was pointed out that the data provided to prove systemic availability were considered inconclusive due to the inconsistency of the data.

On 6 January 2015 the applicant has submitted additional data on the representative substance maltol [FL-no: 07.014] in response to this EFSA evaluation. This additional data regards a study intended to look at systemic exposure of rats following oral administration of this substance, using the same dosing regimen employed in the combined micronucleus and comet test previously submitted.

Terms of Reference as provided by the European Commission

The European Commission requests the European Food Safety Authority (EFSA) to evaluate this new information and, depending on the outcome, proceed to the full evaluation on this flavouring substance in accordance with Commission Regulation (EC) N° 1565/2000[4].

[1] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34-50

[2] EC (European Commission), 2012. Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p. 1-161

[3] EFSA Journal 2014;12(2):3587.

[4] Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. OJ L 180, 19.7.2000, p. 8-16

2. Data and Methodologies

2.1. History of the evaluation of FGE.19 substances

Flavouring Group Evaluation (FGE) 19 (FGE.19) contains 360 flavouring substances from the European Union (EU) Register being α,β-unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation (EFSA, 2008a).

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a). The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The α,β-unsaturated carbonyls were subdivided into subgroups on the basis of structural similarity (EFSA, 2008a). In an attempt to decide which of the substances could go through the Procedure, a (quantitative) structure–activity relationship ((Q)SAR) prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI-MultiCASE Models and ISS-Local Models, (Gry et al., 2007)).

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these α,β-unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007a, b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. Based on these data the Panel decided that 15 subgroups (1.1.1, 1.2.1, 1.2.2, 1.2.3, 2.1, 2.2, 2.3, 2.5, 3.2, 4.3, 4.5, 4.6, 5.1, 5.2 and 5.3) (EFSA, 2008b) could not be evaluated through the Procedure because of concerns with respect to genotoxicity. Corresponding to these subgroups, 15 Flavouring Group Evaluations (FGEs) were established: FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225.

For 11 subgroups the Panel decided, based on the available genotoxicity data and (Q)SAR predictions, that a further scrutiny of the data should take place before requesting additional data from the Flavour Industry on genotoxicity. These subgroups were evaluated in FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220. For the substances in FGE.202, 214 and 218 it was concluded that a genotoxic potential could be ruled out and accordingly these substances will be evaluated using the Procedure. For all or some of the substances in the remaining FGEs, FGE.201, 203, 210, 212, 213, 216, 217 and 220 the genotoxic potential could not be ruled out.

To ease the data retrieval of the large number of structurally related α,β-unsaturated substances in the different subgroups for which additional data are requested, EFSA worked out a list of representative substances for each subgroup (EFSA, 2008c). Likewise, an EFSA genotoxicity expert group has worked out a test strategy to be followed in the data retrieval for these substances (EFSA, 2008b).

The Flavour Industry has been requested to submit additional genotoxicity data according to the list of representative substances and test strategy for each subgroup.

The Flavour Industry has now submitted additional data and the present FGE concerns the evaluation of these data requested on genotoxicity.

2.2. History of the evaluation of the substances belonging to FGE.213

In the EFSA Opinion 'List of α,β-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing' (EFSA, 2008c), representative flavouring substances have been selected for FGE.19 subgroup 2.7, corresponding to FGE.213.

In the first scientific opinion on FGE.213 (EFSA, 2009), the Panel concluded that, based on the data available, the concern with respect to genotoxicity could be ruled out for 11 substances [FL-nos: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 7.117, 07.118, 07.119, 07.120 and 07.168]. Nine of these substances have been evaluated by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) before 2000 to be of no safety concern and, in accordance with Commission Regulation (EC) No 1565/2000, no further consideration is requested. The remaining two substances, ethyl maltol [FL-no: 07.047] and 2-hydroxypiperitone [FL-no: 07.168], were evaluated in FGE.83Rev1 (EFSA CEF Panel, 2010) and FGE.11Rev2 (EFSA CEF Panel, 2011), respectively, using the Procedure.

For maltol [FL-no: 07.014], the Panel requested a combined *in vivo* micronucleus and comet assay in order to clarify the genotoxic potential. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

For the remaining 13 substances [FL-nos: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] additional data on genotoxicity were required for the representative substances, according to the Opinion of the CEF Panel on the 'Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19' (EFSA, 2008b).

FGE	Adopted by EFSA	Link	No. of Substances
FGE.213	27 November 2008	http://www.efsa.europa.eu/en/efsajournal/pub/879.htm	26
FGE.213Rev1	10 April 2014	http://www.efsa.europa.eu/it/efsajournal/pub/3661.htm	26
FGE.213Rev2	09 September 2015	http://www.efsa.europa.eu/it/efsajournal/pub/4244.htm	26

In FGE.213 Revision 1 (FGE.213Rev1) the Panel evaluated additional genotoxicity data submitted by the Flavouring Industry (IOFI, 2012, 2013) in response to a data request presented in FGE.213 (EFSA, 2009).

The new data submitted concerned five of the original six representative substances requested by the Panel (EFSA, 2008c), namely β-ionone [FL-no: 07.008], maltol [FL-no: 07.014], β-damascone [07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] (Table 1).

The Flavour Industry informed that it no longer supports the representative flavouring substance, piperitenone oxide [FL-no: 16.044], for which the Panel requested additional data. However, since piperitenone oxide was a self-representative substance, this did not affect the evaluation of the remaining substances in FGE.213Rev1.

In FGE.213Rev1, one additional substance was included in subgroup 2.7, tr-1-(2,6,6-trimethyl-1-cyclohexen-1-yl)but-2-en-1-one [FL-no: 07.224], which is structurally related to the other substances for which the genotoxic potential could not be ruled out.

In FGE.213Rev1, the Panel concluded that the *in vitro* and *in vivo* genotoxicity data for the selected representative substances β-ionone [FL-no: 07.008], β-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] do not indicate a genotoxic potential. Therefore, these substances, and the nine substances that they represent [FL-no: 02.106, 07.010, 07.041, 07.108, 07.127, 07.136, 07.200, 07.224 and 09.305] could be evaluated through the Procedure.

During the evaluation of *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127], using the Procedure (in FGE.57Rev1), the Panel noted that the chemical structure of *p*-mentha-1,4(8)-dien-3-one [FL-no:

07.127] is actually more closely related to the structure of pulegone (Table 2) than to the structures used for the read-across approach in FGE.213Rev1. New information (NTP, 2011) was found on genotoxicity and carcinogenicity of pulegone, from which additional data are expected to be provided by the applicant. The data available, at present, on pulegone and on the structurally related *p*-mentha-1,4(8)-dien-3-one [FL-no 07.127] do not rule out the concern for genotoxicity and carcinogenicity. Therefore, the genotoxicity of *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] will be reconsidered based on additional data.

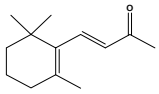
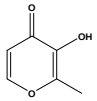
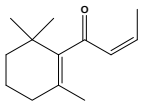
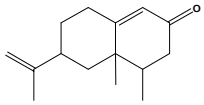
For maltol [FL-no: 07.014], a combined bone marrow micronucleus test and comet assay in rats (Beevers, 2013a) was evaluated by the Panel in FGE.213Rev1. The results of the micronucleus test showed that there were no statistically significant increases in micronucleus frequency for any dose group after oral treatment with maltol when compared with the vehicle control group. The comet assay did not reveal cytotoxicity, necrosis or apoptosis in the hepatocytes as assessed by cloud and halo analysis, and the groups treated with maltol showed mean percentage tail intensities and tail moments that were similar to vehicle controls and fell within historical control ranges.

The Panel noted that, at the dose levels selected, no clinical signs and no bone marrow toxicity were observed in any animal in the maltol-treated groups, which may reflect the possibility that the bone marrow and the liver were not exposed. Therefore, plasma analysis for proof of exposure was requested. Analysis of maltol in plasma was performed using a gas chromatography with mass selective detection (GC–MSD) method. Results showed marked inconsistencies among animals and between sampling times. The Panel concluded that negative findings observed in the combined bone marrow micronucleus test and comet assay in the liver of treated rats could not rule out the concern for genotoxicity of maltol since the data provided to prove systemic availability were considered inconclusive due to the inconsistency of the data.

Following the Panel's conclusion in FGE.213Rev1, the Flavour Industry has submitted a new plasma analysis (Beevers, 2015) performed on the same strain of rats and using the same dosing regimen of the combined micronucleus test and comet assay (Beevers, 2013a). These new data are evaluated in this revision of FGE.213, FGE.213Rev2.

The new data provided show that detectable levels of maltol were found in all plasma samples isolated at 0.5, 1 and 2 hours after dosing. Peak plasma levels of maltol were seen in the majority of animals at 0.5 hours after dose administration. The concentration of maltol detected in plasma was different between the animals of 2 separate cages and the authors of the study did not identify any technical reasons that could account for this difference. The new data submitted are described and evaluated in Section 3 of the present revision. Sections 2.4 and 2.5 report the same information that was present in FGE.213 and FGE.213Rev1, respectively.

Table 1: Representative substances for subgroup 2.7 of FGE.19

FL-no JECFA-no	EU Register name	Structural formula	Comments
07.008 **389**	β-Ionone		*In vitro* assays in bacteria and mammalian cells submitted
07.014 **1480**	Maltol		*In vitro* assays in bacteria and mammalian cells and an *in vivo* combined comet and micronucleus assay submitted
07.083 **384**	β-Damascone		*In vitro* assays in bacteria and mammalian cells and an *in vivo* combined comet and micronucleus assay submitted
07.089 **1398**	Nootkatone		*In vitro* assays in bacteria and mammalian cells submitted

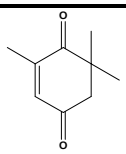
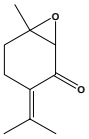
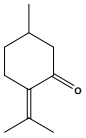
FL-no JECFA-no	EU Register name	Structural formula	Comments
07.109 1857	2,6,6-Trimethylcyclohex-2-en 1,4-dione		*In vitro* assays in bacteria and mammalian cells submitted
16.044 1574	Piperitenone oxide		No longer supported by the Flavour Industry and no data submitted

Table 2: Supporting substance for subgroup 2.7 of FGE.19

FL-no JECFA-no	Substance name	Structural formula	Comments
Not in Register 753	Pulegone		Additional *in vitro* and *in vivo* data (NTP, 2011)

2.3. Presentation of the substances in flavouring group evaluation 213

2.3.1. Description

The Flavouring Group Evaluation 213 (FGE.213) concerned 26 substances (Table 4), corresponding to subgroup 2.7 of FGE.19. Twenty-three of the substances are α,β-unsaturated alicyclic ketones [FL-nos: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] (one substance [FL-no 16.044] is no longer supported by the Flavour Industry and one new substance [FL-no 07.224] has been included in Revision 1) and three are precursors for such ketones [FL-nos: 02.106, 09.305 and 09.525]. Two of these substances [FL-nos: 02.106 and 09.305] are precursors of the ketone β-ionone [FL-no: 07.008] and one [FL-no: 09.525] is a precursor of the ketone maltol [FL-no: 07.014]. Ten of the ketones have the possibility for keto–enol tautomerism [FL-nos: 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168]. Based on experimental evidence for other diketones it is anticipated that the enol is the predominant form.

Twenty-two of the substances in FGE.213 (including the new substance [FL-no 07.224], excluding [FL-no 16.044]) have formerly been evaluated by the JECFA (JECFA, 1999, 2001, 2006a, b, 2009a), a summary of their current evaluation status by the JECFA is given in Table 5.

As the α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a) the available data on genotoxic or carcinogenic activity for the 26 unsaturated alicyclic ketones and precursor in subgroup 2.7 will be considered in this FGE.

The Panel has also taken into consideration the outcome of the predictions from five selected (Q)SAR models (Benigni and Netzeva, 2007a; Gry et al., 2007; Nikolov et al., 2007) on 22 ketones [FL-nos: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168 and 07.200]. The 22 ketones and their (Q)SAR predictions are shown in Table 6.

2.4. Data evaluated by the Panel in FGE.213[5]

2.4.1. (Q)SAR predictions

In Table 6 the outcomes of the (Q)SAR predictions for possible genotoxic activity in five *in vitro* (Q)SAR models (ISS-Local Model–Ames test, DTU–NFI-MULTICASE–Ames test, Chromosomal aberration test (using Chinese hamster ovary (CHO) cells), Chromosomal aberration test (using Chinese hamster lung (CHL) cells) and Mouse lymphoma test) are presented.

Maltol [FL-no: 07.014], ethyl maltol [FL-no: 07.047] and nootkatone [FL-no: 07.089] were predicted positive with the MultiCASE model on chromosomal aberrations in CHL cells. All other predictions were negative or the substances were out of domain.

2.4.2. Genotoxicity studies

In subgroup 2.7 there are studies available for four substances. For maltol [FL-no: 07.014] eight *in vitro* and three *in vivo* studies have been evaluated. For ethyl maltol [FL-no: 07.047] two *in vitro* and one *in vivo* study were evaluated. Two *in vitro* studies concerning β-ionone [FL-no: 07.008] and one *in vitro* study for 3-methylcyclopentan-1,2-dione [FL-no: 07.056] were evaluated.

Study validation and results are presented in Tables 7 and 8.

In studies that were considered valid, the following results were obtained:

- Maltol-induced gene mutations in bacteria (Bjeldanes and Chew, 1979) and sister chromatid exchanges (SCEs) in human lymphocytes (Jansson et al., 1986). *In vivo*, maltol-induced micronuclei in mouse bone marrow after intraperitoneal application (Hayashi et al., 1988). Negative results were obtained in a sex-linked recessive lethal mutation assay in *Drosophila* (Mason et al., 1992). However, the micronucleus assay is considered more relevant than the *Drosophila* assay.
- Ethyl maltol-induced gene mutations in bacteria (Bjeldanes and Chew, 1979).
- A negative result was obtained with β-ionone in a gene mutation assay in bacteria (Mortelmans et al., 1986).

The validity of other studies was limited or could not be evaluated.

2.4.3. Carcinogenicity studies

In a combined study of developmental toxicity and carcinogenicity, three successive generations of male and female Charles River CD-COBS rats received 3-ethyl-2-hydroxy-2-cyclopenten-1-one (owing to keto–enol tautomerism this substance can exist as two isomers; the keto-isomer is 3-ethylcyclopentan-1,2-dione [FL-no: 07.057], a synonym for the keto-isomer is ethylcyclopentenolone) in the basal diet at doses of 0 (untreated control), 0 (propylene glycol control), 30, 80 or 200 mg/kg body weight (bw) per day. The F1 generation was initially exposed *in utero*, subsequently via the dams' milk until weaning, and then treated for two years and bred twice (at days 99 and 155). In the F1 generation, there were 100 animals of each sex in the untreated control group, 50 animals of each sex in both the propylene glycol control and 3-ethyl-2-hydroxy-2-cyclopenten-1-one-treated groups. Survival, clinical symptoms, food consumption, reproductive performance, haematological and clinical chemistry parameters were not adversely affected. Gross pathological and histopathological examination revealed no significant treatment-related effects. The incidence of benign or malignant tumours in treated animals was similar to that in controls. The No Observed Effect Level (NOEL) was 200 mg/kg bw per day (King et al., 1979).

The Panel concluded that 3-ethyl-2-hydroxy-2-cyclopenten-1-one (3-ethylcyclopentan-1,2-dione [FL-no: 07.057]) was not carcinogenic in rats under the study conditions.

Groups of 25 male and female rats were fed for two years on diets containing ethyl maltol [FL-no: 07.047] calculated to deliver 0, 50, 100 and 200 mg ethyl maltol/kg bw/day. No abnormalities were

[5] The data presented in Section 2.4 are cited from the first version of the present FGE.213. These data are the basis for the conclusions in FGE.213 requesting additional genotoxicity data.

seen as regards survival, clinical appearance, growth rate or food consumption, clinical chemistry, haematology and urinalysis. No histopathological changes and no increases in neoplasms were seen after the treatment with ethyl maltol (Gralla et al., 1969).

Study validation and results are presented in Table 9.

The Panel noted that this study was performed before Organisation for Economic Co-operation and Development (OECD) test guidelines 451/453 (1981a, b) had been established and it does not meet the criteria of these OECD test guidelines with respect to the number of animals. However, the Panel concluded that ethyl maltol was not carcinogenic in rats in this study.

2.4.4. Conclusion on genotoxicity and carcinogenicity

For the substances of this group, the applicability of the (Q)SAR models is very limited since many substances were out of domain in the ISS model and the MultiCASE models.

Two substances [FL-nos: 02.106 and 09.305] are precursors of β-ionone [FL-no: 07.008] and therefore, the conclusions for these two precursors could be based on the conclusions drawn for the corresponding ketone [FL-no: 07.008]. Maltyl isobutyrate [FL-no: 09.525] is a precursor of maltol [FL-no: 07.014], and accordingly, the conclusion for maltyl isobutyrate could be based on the conclusion drawn for maltol.

Maltol and ethyl maltol were considered separately because, in contrast to the other substances in this subgroup, they contain a ring-oxygen atom.

A carcinogenicity study on ethyl maltol [FL-no: 07.047] in rats (Gralla et al., 1969) has been evaluated. Although there were fewer animals per group than that suggested in OECD guidelines 451/453 (1981a, b), the study was in accordance with the standards available at that time. The Panel concluded that the result from this study could overrule the mutagenicity observed with ethyl maltol in bacteria, but not the mutagenicity observed with maltol [FL-no: 07.014] *in vitro* and *in vivo*. Since the micronuclei induced by maltol in mice were analysed after intraperitoneal application, a micronucleus assay after oral application is required, in addition to an *in vivo* comet assay, in order to clarify the genotoxic potential of maltol. A combination of the micronucleus assay and the comet assay in a single study would also be acceptable. The results of these assays would also be applicable to maltyl isobutyrate [FL-no: 09.525], which is a precursor of maltol.

No carcinogenicity was observed with 3-ethyl-2-hydroxy-2-cyclopenten-1-one [FL-no: 07.057] in rats. This substance was considered representative for nine substances [FL-nos: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080]. Therefore, the Panel concluded that the structural alert for genotoxicity is overruled for 3-ethyl-2-hydroxy-2-cyclopenten-1-one [FL-no: 07.057] as well as for the nine structurally related substances.

For the 13 remaining substances (including two precursors of a ketone) [FL-nos: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] a genotoxic potential could not be ruled out since only one valid negative bacterial genotoxicity study on [FL-no: 07.008] is available for these substances.

2.4.5. Conclusion based on the data available to the Panel in FGE.213

The Panel concluded that ethyl maltol [FL-no: 07.047], 3-ethylcyclopentan-1,2-dione [FL-no: 07.057] and the nine structurally related substances [FL-nos: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080] can be evaluated through the Procedure.

For maltol [FL-no: 07.014], a micronucleus assay after oral application is required, in addition to an *in vivo* comet assay, in order to clarify the genotoxic potential. A combination of the micronucleus assay and the comet assay in a single study would also be acceptable. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

At present, the remaining 13 substances (including two precursors of a ketone) [FL-nos: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] cannot be evaluated using the Procedure. Additional data on genotoxicity are requested for the representative substances of this subgroup according to the opinion of the Panel on the 'Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19' (EFSA, 2008b).

2.5. Additional genotoxicity data evaluated by the Panel in FGE.213Rev1[6]

2.5.1. Presentation of the additional data

Based on Panel requirements published in FGE.213 (EFSA, 2009), additional data have been provided by the Flavour Industry for the representative substances: β-ionone [FL-no: 07.008], maltol [FL-no: 07.014], β-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109].

FGE.213, Revision 1 (FGE.213Rev1), includes the assessment of these additional genotoxicity studies (Table 3).

Table 3: Studies evaluated in FGE.213Rev1

Substance/study type	Bacterial Reverse Mutation assay	*In Vitro* Micronucleus test	*In Vivo* combined Micronucleus test and Comet assay
β-Ionone [FL-no: 07.008]	Ballantyne, 2011	Stone, 2011a	
Maltol [FL-no: 07.014]	Ballantyne, 2012	Whitwell, 2012	Beevers, 2013a
β-Damascone [FL-no: 07.083]	Bowen, 2011b	Stone, 2012	Beevers, 2013b, c
Nootkatone [FL-no: 07.089]	Marzin, 1998	Stone, 2011b	
2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]	Bowen, 2011a	Lloyd, 2011	

2.5.2. *In vitro* data

Bacterial reverse mutation assay

β-Ionone [FL-no: 07.008]

β-Ionone [FL-no: 07.008] was tested in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Ballantyne, 2011). In the first experiment, the concentrations used were 0.32, 1.6, 8, 40, 200, 1 000 and 5 000 µg/plate of β-ionone and the plate incorporation methodology was used. Toxicity ranging from slight thinning of the background lawn to complete killing of the tester strains was observed at 1 000 and/or 5 000 µg/plate for all tester strains in the absence and presence of S9-mix. In the second experiment, the concentrations tested were 10.24, 25.6, 64, 160, 400 and 1 000 µg/plate and the treatments in the presence of S9-mix used the pre-incubation method. Toxicity ranging from thinning of the background lawn and/or reduction in revertant numbers to complete killing of the tester bacteria occurred in all strains at 1 000 µg/plate in the absence and presence of S9-mix, and was also seen down to 160 and/or 400 µg/plate for some individual strains. The study design complied with current recommendations and an acceptable highest concentration was achieved. There was clearly no evidence of any mutagenic effect induced by β-ionone in any of the strains, either in the absence or presence of S9-mix.

Maltol [FL-no: 07.014]

Maltol [FL-no: 07.014] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Ballantyne, 2012). In the first experiment, the concentrations were 0.32, 1.6, 8, 40, 200, 1 000 and 5 000 µg/plate of maltol and the plate incorporation methodology was used. Toxicity in the form of reduction of the number of revertants in the tester strain TA102 was observed at concentrations of 200 µg/plate and greater in the presence of

[6] The data presented in Section 2.5 are cited from FGE.213Rev1. These data are the basis for the conclusions in FGE.213Rev1 requesting additional data.

S9-mix and 1 000 and 5000 µg/plate in the absence of S9-mix. In the second experiment, the concentrations were 51.2, 128, 320, 800, 2 000 and 5 000 µg/plate and the treatments in the presence of S9-mix used the pre-incubation method in all strains. In tester strain TA102 an additional lower concentration of 20.48 µg/plate was incorporated into the testing protocol in both the absence and presence of S9-mix to assess, more carefully, the toxicity observed in experiment 1. Toxicity in the form of thinning of the background lawn and/or reduction in numbers of revertants occurred at 5 000 µg/plate in strain TA102 in the absence and presence of S9-mix, and in strain TA100 only in the presence of S9-mix. The study design complied with current recommendations and an acceptable highest concentration was achieved. There was no evidence of any mutagenic effect induced by maltol in any of the strains, either in the absence or presence of S9-mix.

β-Damascone [FL-no: 07.083]

An Ames assay was conducted in *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA102 to assess the mutagenicity of β-damascone (purity: 95 %), both in the absence and in the presence of metabolic activation by S9-mix, in three separate experiments (Bowen, 2011b). The assay was performed according to OECD Guideline 471 (1997a) and according to Good Laboratory Practice (GLP) principles.

An initial experiment was carried out both in the absence and presence of S9-mix activation in all five strains, using final concentrations of β-damascone between 0.32 and 5000 µg/plate (0.32, 1.6, 8, 40, 200, 1 000, 5 000 µg/plate), plus negative (solvent) and positive controls. Evidence of toxicity was observed through thinning of the background lawn to complete killing at concentrations of 1 000 µg/plate and greater for strains TA1535, TA1537 and TA102 and/or 5000 µg/plate for strains TA98 and TA100 in the absence and presence of S9-mix. In the second experiment the highest concentration was retained for strains TA98 and TA100 in the absence and presence of S9-mix. In all other tester strains, the highest concentration was reduced to 2500 µg/plate based on toxicity observations. In addition, more narrow concentration intervals were used, starting at either 78.13 µg/plate or 156.3 µg/plate (78.13, 156.3, 312.5, 625, 1250, 2500 µg/plate). The standard plate incorporation assay was used in the first experiment but a pre-incubation step with S9-mix activation treatment was added in the second experiment to increase the chance of detecting a positive response. Evidence of toxicity was observed in TA98 at 625 µg/plate (in the presence of S9-mix) and at concentrations of 1250 µg/plate and greater (in the absence of S9-mix). Evidence of toxicity was observed in strains TA1535, TA1537 and TA102 at 625 µg/plate in the absence and presence of S9-mix. In strain TA100 toxicity was observed at concentrations of 1250 µg/plate and greater (in the presence of S9-mix) and at concentrations of 2500 µg/plate and greater (in the absence of S9-mix).

The third experiment was conducted using strain TA98 in the presence of S9-mix activation using the pre-incubation method. The maximum test concentration was reduced to 1 250 µg/plate based on toxicity observed in the previous experiments. In addition, more narrow concentration intervals were used, covering 19.53 to 1 250 µg β-damascone/plate (19.53, 39.06, 78.13, 156.3, 312.5, 625 and 1 250 µg/plate). Evidence of toxicity was observed at the highest four concentrations in strain TA98 in the presence of S9-mix. In all three experiments, no statistically significant increases in revertant numbers were observed at any concentration, in any of the strains, either in the presence or absence of S9-mix activation.

The Panel agreed with the conclusion of the study authors that β-damascone did not induce mutations in five strains of *S. typhimurium*, when tested under the conditions of this study.

Nootkatone [FL-no: 07.089]

Nootkatone [FL-no: 07.089] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence or presence of S9-mix (Marzin, 1998). A preliminary toxicity test to identify appropriate concentrations for the mutagenicity assays was performed in the absence and presence of S9-mix, and cytotoxicity was observed at 50 µg/plate in the absence of S9-mix and at 150 µg/plate in the presence of S9-mix. In the first mutagenicity experiment using plate incorporation methodology the concentrations tested were 0.5, 1.5, 5, 15 and 50 µg/plate in the absence of S9-mix metabolic activation and 1.5, 5, 15, 50 and 150 µg/plate in the presence of S9-mix. In the second experiment the plate incorporation method was used in the absence of S9-mix and the concentrations were 0.5, 1.5, 5, 15 and 50 µg/plate. While the pre-incubation method was used in the presence of S9-mix and

the concentrations were 0.5, 1.5, 5, 15, 50 and 150 µg/plate. Thus, the study design complied with current recommendations and an acceptable highest concentration was achieved. There was no evidence of any mutagenic effect induced by nootkatone in any of the strains, either in the absence or presence of S9-mix.

2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]

2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Bowen, 2011a). In the first experiment, the concentrations tested were 0.32, 1.6, 8, 40, 200, 1 000 and 5 000 µg/plate and plate incorporation methodology was used. In the second experiment, the concentrations were 156.3, 312.5, 625, 1 250, 2 500 and 5 000 µg/plate of 2,6,6-trimethylcyclohex-2-en-1,4-dione and treatments in the presence of S9-mix used the pre-incubation method. The test chemical elicited evidence of cytotoxicity in the form of background lawn thinning or marked reduction of the number of revertants in experiment 1 at 1 000 and/or 5 000 µg/plate in strains TA102 and TA1535 in the presence of S9-mix and in experiment 2 at 2 500 and/or 5 000 µg/plate in strain TA102 in the absence and presence of S9-mix. Thus, the study design complied with current recommendations and an acceptable highest concentration was achieved. There was no evidence of any mutagenic effect induced by 2,6,6-trimethylcyclohex-2-en-1,4-dione in any of the strains, either in the absence or presence of S9-mix.

Summary of the bacterial reverse mutation assay for all the substances is reported in Table 10.

Micronucleus Assay

β-Ionone [FL-no: 07.008]

β-Ionone [FL-no: 07.008] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat liver S9-mix fraction as an *in vitro* metabolising system. Cells were stimulated for 48 hours with phytohaemagglutinin (PHA) to produce exponentially growing cells and then treated for 3 hours (followed by a 21-hour recovery period) with 0, 30, 50 or 60 µg/ml of β-ionone in the absence of S9-mix and 0, 80, 100 or 120 µg/ml in the presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the highest concentrations were 52 % and 59 %, respectively.

In a parallel assay, cells were treated for 24 hours with 0, 5, 15 and 17.50 µg/ml of β-ionone in the absence of S9-mix with no recovery period. The highest concentration induced 58 % cytotoxicity. There were 2 replicate cultures per treatment and 1000 binucleate cells per replicate were scored for micronuclei. Thus, the study design complies with current recommendations (OECD Guideline 487), and acceptable levels of cytotoxicity were achieved at the highest concentrations used in all parts of the study. Treatment of cells with β-ionone for 3 hours with a 21-hour recovery period showed an increase in the frequency of micronucleated binucleated (MNBN) cells in one single replicate at concentrations of 30 and 120 µg/ml (0.9 % and 1.5 %, respectively) in the absence and presence of S9-mix, respectively. At 30 µg/ml, the lowest concentration tested in the absence of S9-mix, the increase in the frequency of MNBN cells was slightly above the 95 % confidence interval of the historical control range (0.2–0.8 %). In addition, in the presence of S9-mix, one replicate of the lowest concentration tested (80 µg/ml) had an increase in the frequency of MNBN cells at the upper limit of the 95 % confidence interval of the historical control range (0.10–1.10 %) but did not reach statistical significance. To ensure that these single occurrences are random an additional 1 000 binucleate cells were scored from the concurrent controls, 80 and 120 µg/ml cultures. The scoring of further cells resulted in overall mean frequencies of MNBN cells that were not significantly different from concurrent controls and fell below the upper 95 % confidence interval of the normal control range (recalculated due to change of stain), and therefore showed that the earlier increases were due to chance. It was concluded that β-ionone [FL-no: 07.008] did not induce micronuclei up to toxic concentrations when assayed in cultured human peripheral lymphocytes for 3 + 21 hours in the absence and presence of S9-mix or when incubated for 24 + 0 hours in the absence of S9-mix (Stone, 2011a).

Maltol [FL-no: 07.014]

Maltol [FL-no: 07.014] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat liver S9-mix fraction as an *in vitro* metabolising system (Whitwell, 2012). Cells were stimulated for 48 hours with PHA to produce exponentially growing cells and then treated for 3 hours (followed by a 21-hour recovery period) with 0, 400, 800 or 1 262 µg/ml of maltol, the last being equivalent to 10 mM, in the absence and presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the highest concentrations were 24 % and 19 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 125, 200 and 300 µg/ml of maltol in the absence of S9-mix with no recovery period. The highest concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment, and 1 000 binucleate cells per replicate (i.e. 2 000 cells per concentration) were scored for micronuclei. Thus, the study design complies with current recommendations (OECD Guideline 487), and acceptable highest concentrations, either 10 mM or 50–60 % toxicity, were achieved in all parts of the study. A statistically significant increase in the occurrence of MNBN cells was observed following 3 + 21 hours treatment in the presence of S9-mix at the two highest concentrations scored. Statistically significant and concentration-dependent increases in MNBN cells were seen in the 3 + 21 hours treatment groups in the absence of S9-mix, but it was noted that the increases at the two highest concentrations scored exceeded historical control ranges in only one of the two replicate cultures. No increases were observed in the frequency of MNBN cells in those that had received continuous (24 + 0 hours) treatment, but due to the cytotoxicity of maltol, lower concentrations were analysed. To further investigate these observations, fluorescence *in situ* hybridisation (FISH) analysis using human pan-centromeric probes was conducted to assess whether the mechanism of action could be attributed to chromosome loss (aneuploidy) or chromosome breakage (clastogenicity). Slides were prepared from the two highest concentrations (800 and 1 262 µg/ml) in the absence and presence of S9-mix. The FISH analysis revealed that following maltol treatment the majority (69–76 %) of micronuclei did not contain a centromere. The Panel concluded that maltol induced micronuclei *in vitro* in cultured human peripheral blood lymphocytes in the presence of rat liver metabolic activation (S9-mix) via a clastogenic mechanism of action (Whitwell, 2012). However, the Panel considered that the results observed in the absence of S9-mix were equivocal because of the fact that the increases observed (which were statistically significantly different from concurrent solvent control) were not reproduced in replicate cultures.

β-Damascone [FL-no:07.083]

β-Damascone (purity: 95 %) was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence or absence of rat S9 fraction as an *in vitro* metabolising system (Stone, 2012). Cells were stimulated for 48 hours with PHA to produce exponentially growing cells and then treated for 3 hours (followed by a 21-hour recovery period) with concentrations ranging from 2 to 30 µg/ml. For the treatment of 3 hours with a 21-hour recovery period, the concentrations of β-damascone at 8, 16 and 22 µg/ml or at 12, 16, 18 µg/ml were retained for micronuclei (MN) numeration, in the absence or in the presence of S9-mix respectively. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 59 % and 51 %, respectively. Thus, the study design complies with OECD Guideline 487 and follows GLP principles.

In a parallel assay, cells were treated for 24 hours (with no recovery period) in the absence of S9-mix with concentrations ranging from 1 to 15 µg/ml, and the concentrations of 6, 8 and 9 µg/ml of β-damascone were retained for MN numeration. The highest concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment and 1 000 binucleate cells per replicate were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487, 2010), and acceptable levels of cytotoxicity were achieved at the highest concentrations used in all parts of the study.

Treatment of cells with β-damascone for 3 + 21 hours in the presence of S9-mix showed a statistically significant concentration-dependent increase in the induction of MNBN cells with 0.55, 2.10 and 2.70 % MNBN cells versus 0.35 % in the concurrent control and 0.1 to 1.1 % for the historical controls.

Treatment of cells with β-damascone for 3 + 21 and 24 + 0 hours in the absence of S9-mix resulted in sporadic increases in MNBN frequency. These increases were only observed in single replicates and were not concentration-related. Therefore, the effect of β-damascone was further investigated through the scoring of additional cells (2 more replicates of 1000 cells each) from the affected concentrations and concurrent controls.

Treatment of cells, in the absence of S9-mix, for 3 + 21 hours induced a statistically significant increase in the frequency of MNBN cells at 8 and 22 µg/ml (0.80 % and 0.93 %, respectively) compared with concurrent control (0.38 %), but not at the mid-dose of 16 µg/ml (0.53 % MNBN cells). The frequency of MNBN cells exceeded the historical controls (0.2–0.8 %) in 3 out of 4 replicates at the highest concentration tested (22 µg/ml). Treatment of cells for 24 hours with no recovery period in the absence of S9-mix showed a statistically significant increase in the frequency of MNBN cells at the mid-dose of 8 µg/ml (0.95 % MNBN cells) when compared with concurrent control (0.40 %) with no correlation to concentration. The frequency of MNBN cells exceeded the historical controls (0–1.1 %) in only one replicate at 8 µg/ml.

The authors considered that this result reaffirmed the sporadic nature of the induction of MNBN cells in the absence of S9-mix. It was concluded that the treatment with β-damascone for 3 + 21 hours or 24 + 0 hours (in the absence of S9-mix) induced sporadic increases in MNBN cells when compared with concurrent controls and not concentration-related; therefore, the results were considered equivocal. In the same test system, β-damascone induced micronuclei in cultured human peripheral blood lymphocytes following 3 + 21 hours treatment in the presence of S9-mix (Stone, 2012). The Panel noted that after the new reading of slides the increase in the frequency of MNBN cells was still statistically significant even at weak cytotoxic levels.

Therefore, the Panel concluded that β-damascone is genotoxic in the *in vitro* micronucleus assay on human lymphocytes with metabolic activation and equivocal without metabolic activation.

Nootkatone [FL-no: 07.089]

Nootkatone [FL-no: 07.089] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9-mix fraction as an *in vitro* metabolising system (Stone, 2011b). Cells were stimulated for 48 hours with PHA to produce exponentially growing cells and then treated for 3 hours (followed by a 21-hour recovery period) with 0, 50, 70 or 80 µg/ml of nootkatone in the absence of S9-mix and 0, 160, 180 and 185 µg/ml in the presence of S9-mix, respectively. The levels of cytotoxicity (reduction in replication index) at the highest concentrations were 60 % and 58 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 10, 15, 22 and 24 µg/ml of nootkatone in the absence of S9-mix with no recovery period. The highest concentration induced 62 % cytotoxicity. There were 2 replicate cultures per treatment and 1 000 binucleate cells per replicate (i.e. 2 000 cells per dose) were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487) and acceptable levels of cytotoxicity were achieved at the highest concentrations used in all parts of the study. No evidence of chromosomal damage or aneuploidy was observed as frequencies of MNBN cells were not significantly different from concurrent controls and fell within historical control ranges for all treatments with nootkatone in the presence or absence of S9-mix metabolic activation (Stone, 2011b).

2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]

2,6,6-Trimethylcyclohex-2-en-1,4-dione was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9-mix fraction as an *in vitro* metabolising system (Lloyd, 2011). Cells were stimulated for 48 hours with PHA to produce exponentially growing cells and then treated for 3 hours (followed by a 21-hour recovery period) with 0, 500, 1 000 or 1 522 µg/ml of 2,6,6-trimethylcyclohex-2-ene-1,4-dione in the absence of S9-mix and 0, 1 000, 1 250 and 1 522 µg/ml in the presence of S9-mix, the highest concentration being equivalent to 10 mM. The levels of cytotoxicity (reduction in replication index) at the highest concentrations were 3 % and 9 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 300, 420 and 550 µg/ml of 2,6,6-trimethylcyclohex-2-ene-1,4-dione in the absence of S9-mix with no recovery period. The highest concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment and

1 000 binucleate cells per replicate (i.e. 2 000 cells per concentration) were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487), and acceptable highest concentrations, either 10 mM or 50–60 % toxicity, were achieved in all parts of the study. No evidence of chromosomal damage or aneuploidy was observed as frequencies of MNBN cells were not significantly different from concurrent controls and fell within historical ranges for all 2,6,6-trimethylcyclohex-2-ene-1,4-dione treatments in the presence or absence of S9-mix metabolic activation (Lloyd, 2011).

The results of *in vitro* micronucleus studies are summarised in Table 10.

2.5.3. Genotoxicity *in vivo* data

In vivo Combination Assay (comet + micronucleus tests)

Since no positive results were seen in either the bacterial mutation assay or *in vitro* micronucleus tests with β-ionone [FL-no: 07.008], nootkatone [FL-no: 07.089] or 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109], no *in vivo* follow-up testing was required. To address the effects seen in the *in vitro* micronucleus assay with maltol [FL-no: 07.014] and β-damascone [FL-no: 07.083] a combination assay comprising a liver comet assay and an *in vivo* micronucleus assay in rats, after oral application, was performed to further assess the genotoxic potential for both substances. The results are summarised in Table 11.

Maltol [FL-no: 07.014]

Maltol was evaluated in an *in vivo* bone marrow micronucleus assay and liver comet assay in male Han Wistar (HsdHan:WIST) rats, with 6 rats per dose group (Beevers, 2013a). The rats were administered 3 doses of 70, 350 and 700 mg/kg bw of maltol by oral gavage at time 0, 24 and 45 hours. Rats were killed and sampled at 48 hours post the initial dose. The maximum tolerable dose was estimated to be 700 mg/kg bw/day based on a range-finding study where maltol was tested at 360, 500, 700, 1 000, 1 500 and 2 000 mg/kg bw/day. Clinical observations (piloerection, ataxia, bradypnoea) and mortalities were observed at doses of 1000 mg/kg bw/day and greater. For the micronucleus assay 2 000 polychromatic erythrocytes (PCE) per rat were scored. The negative control had a normal, low frequency (0.11 %) of micronucleated polychromatic erythrocytes (MNPCEs) and a ratio of 53.7 % PCE. The positive control group resulted in a significant increase in MNPCEs (1.58 %) accompanied by some bone marrow toxicity (29.57 % PCE). Although an individual rat in the 700 mg/kg maltol dose group showed a frequency of 9 MNPCEs, which resulted in significant heterogeneity in this dose group, this was considered an outlying data point because the other 5 rats in the group were exhibiting normal control-level MNPCE frequencies (Beevers, 2013a). Overall, the results showed that there were no statistically significant increases in micronucleus frequency for any dose group after oral treatment with maltol when compared with the vehicle control group. However, in the main experiment, at the dose levels selected, no clinical signs and bone marrow toxicity were observed in any animal in the maltol-treated groups, which may reflect the possibility that the bone marrow and liver were not exposed.

In order to clarify this issue, the Panel requested plasma analysis for proof of exposure. Plasma was obtained from two satellite groups of male animals (3 + 3 animals) dosed with maltol by oral gavage at 700 mg/kg bw/day, during conduction of the main study (Beevers, 2013a). Plasma obtained from 0.5 ml blood drawn from the jugular vein from each animal was frozen in the event that analysis for proof of exposure and toxicokinetics were required. All doses of maltol were given as three administrations, at 0, 24 and 45 hours. Three samples of plasma were obtained from one group of animals at 0.5, 2 and 8 hours and three samples from the other group at 1, 4 and 24 hours from the last administration. Analysis of maltol in plasma was performed using a gas chromatography with mass selective detection (GC-MSD) method. From an analytical point of view, the Panel considered the employed approach, which was based on the use of ethylmaltol as an internal standard, as sufficient. Results showed a marked inconsistency between sampling times and animals. In samples collected at 0.5, 2 and 8 hours from last administration maltol was found in 2 out of 3 satellite animals at plasma concentrations of 265–283 µg/ml after 0.5 and 2 hours, but no longer detectable after 8 hours. In the plasma of the third animal maltol was not detectable at any time. On the other hand, in samples from another animal group (n = 3) collected at 1, 4 and 24 hours from last administration, maltol was

found at levels of 75–106 µg/l after 1 hour in all 3 animals and no longer detectable after 4 and 24 hours (Mallinson and Hough, 2014). The authors concluded that the results obtained provided evidence that maltol is present in plasma shortly after dosing. However, the Panel did not agree with this conclusion and it considered the results of the bioanalytical study as inconclusive.

In the combined comet assay, livers of rats were removed at 48 hours after the first dose (i.e. 3 hours after the final dose), cut into small pieces and forced through a bolting cloth. Single cell suspensions were embedded in low melting point agarose on slides and lysed. The DNA was unwound and subjected to electrophoresis at pH > 13 and then neutralised according to standard techniques. For each animal, 100 cells (50 cells/slide from 2 slides) were scored for comets (tail intensity and tail moment) using commercial image analysis equipment.

The comet assay did not reveal cytotoxicity, necrosis or apoptosis in the hepatocytes as assessed by cloud and halo analysis and the groups treated with maltol showed mean percentage tail intensities and tail moments that were similar to vehicle controls and fell within historical control ranges. The positive control group treated with ethyl methanesulphonate showed significant increases in both parameters (Beevers, 2013a).

Considering that maltol has been shown to induce micronuclei in mouse bone marrow after intraperitoneal injection (Hayashi et al., 1988), the Panel concludes that negative findings observed in the combined bone marrow micronucleus test and comet assay in the liver of treated rats could not rule out the concern for genotoxicity for maltol since the data provided to prove systemic availability were considered inconclusive due to the inconsistency of the data.

β-Damascone [FL-no:07.083]

A combined *in vivo* micronucleus assay and comet assay was performed after oral application of β-damascone (purity: 95.6 %) to further assess the genotoxic potential of β-damascone and damascones more generally. The results are summarised in Table 11. β-Damascone was evaluated in an *in vivo* bone marrow micronucleus assay and liver and duodenum comet assay in groups of 6 male Han Wistar (HsdHan:WIST) rats per dose group (Beevers, 2013c). Based on a range-finding study, 500 mg/kg/day was considered an appropriate estimate of the maximum tolerated dose (MTD) because doses of 750 mg/kg/day and greater induced moderate to severe clinical signs of toxicity, which included piloerection, decreased activity, hunched posture and abnormal breathing. The rats were administered 3 doses of 125, 250 and 500 mg/kg bw of β-damascone by oral gavage at time 0, 24 and 45 hours. The rats were sacrificed and sampled at 48 hours post the initial dose.

Animals administered β-damascone showed clear findings during pathological analysis. Hepatocyte vacuolation was present in animals given 500 mg/kg/day, and was characterised by scattered, occasionally shrunken hepatocytes with perinuclear cytoplasmic eosinophilia and peripheral cytoplasmic vacuolation. Single cell necrosis was present in a single animal given 500 mg/kg/day. Single cell necrosis was characterised by death of individual hepatocytes throughout the liver, with limited inflammatory cell involvement. There was a dose-related reduction in the level of glycogen vacuolation in animals given 250 or 500 mg/kg/day. Glycogen vacuolation was characterised by generally perinuclear, clear, variably sized, indistinctly defined, vacuoles. Finally, increased mitosis was present in animals from all groups given β-damascone. The greatest severity was present in animals given 250 mg/kg/day, and the lowest incidence was present in animals given 500 mg/kg/day. Increased mitosis was characterised by an increase, above the normal low background incidence, of mitotic figures within the liver parenchyma. Collectively, these findings indicate that the test animals were systemically exposed to β-damascone.

The negative control had a 0.11 % average rate of MNPCE and a ratio of 50.2 % PCE; the 125 mg/kg β-damascone treatment group had a MNPCE rate of 0.09 % and PCE ratio of 49.17 %; the 250 mg/kg treatment group had a 0.09 % MNPCE rate and 52.30 % PCE ratio; the 500 mg/kg treatment group showed 0.06 % MNPCEs and 37.63 % PCE ratio. The positive control group resulted in 1.54 % MNPCEs and a 43.17 % PCE ratio (Beevers, 2013c). The group mean frequencies observed were similar to concurrent vehicle controls for all dose groups and also were within the historical control values (mean: 0.12 %). There was a reduction in PCE ratio at the highest dose level indicating bone marrow toxicity, which demonstrates target organ exposure. These results showed that there was no statistically significant increase in micronuclei induced with β-damascone under these test conditions when compared with the negative control group. In addition, there were no statistically significant

differences among erythrocyte parameters examined in this study. It was concluded that β-damascone did not induce micronucleated erythrocytes in rat bone marrow cells following administration by oral gavage.

The comet assay in the liver tissue did not reveal cytotoxicity, necrosis or apoptosis in the hepatocytes as assessed by cloud and halo analysis. Hepatocytes of rats dosed with β-damascone were evaluated for percentage tail intensities and tail moments (± standard error of the mean, SEM); the 125 mg/kg β-damascone group had 2.45 ± 0.13 % tail intensity and 0.27 ± 0.02 % tail moment; the 250 mg/kg group had 2.99 ± 0.31 % tail intensity and 0.33 ± 0.03 tail moment; the 500 mg/kg group had 2.93 ± 0.24 % tail intensity and 0.31 ± 0.03 tail moment, which were similar to concurrent vehicle controls (tail intensity of 2.67 ± 0.26 % and 0.29 ± 0.03 tail moment) and fell within the testing laboratories historical control range for vehicle controls (0.3–8.15 % tail intensity and 0.04–0.81 tail moment). The comet arm of this study confirms that β-damascone did not induce DNA damage in the liver under the conditions of this study (Beevers, 2013c).

In a satellite study the slides from the duodenum tissue samples collected in the above study (Beevers, 2013c) were analysed for comet tailing effects (Beevers, 2013b). Duodenum cells of rats dosed with β-damascone were evaluated for percentage tail intensities and tail moments (± standard error of the mean, SEM); the 125 mg/kg β-damascone group had 2.01 ± 0.43 % tail intensity and 0.32 ± 0.03 % tail moment; the 250 mg/kg group had 1.47 ± 0.15 % tail intensity and 0.16 ± 0.02 tail moment; the 500 mg/kg group had 2.03 ± 0.19 % tail intensity and 0.19 ± 0.02 tail moment, which were similar to concurrent vehicle controls (tail intensity of 2.24 ± 0.43 % and 0.23 ± 0.04 % tail moment) and fell within the testing laboratories historical control range for vehicle controls (0.3–8.15 % tail intensity and 0.04–0.81 tail moment). The duodenum comet arm of this study confirms that β-damascone did not induce DNA damage in the duodenum under the conditions of this study. The vehicle control data were within historical control ranges (95 % reference range: 0.77 to 8.32 % for tail intensity and 0.08 to 1.15 % for tail moment) and the positive control induced a clear increase in DNA damage. The study was therefore confirmed as valid. There was no evidence of duodenum toxicity as would be suggested by increases in clouds or halo cells.

The percentage tail intensity and tail moment at all dose levels were very similar to the concurrent vehicle control, thus confirming there is no test article-related DNA damage. The additional tissue sample analysis for comet tailing showed a negative result for this study (Beevers, 2013b).

The results from the combined *in vivo* micronucleus induction study and comet assay show that orally administered β-damascone did not induce micronucleated erythrocytes in rat bone marrow cells nor genotoxic events in liver and duodenum of rats.

2.5.4. Conclusion

Flavouring Group Evaluation 213 concerned 26 substances, corresponding to subgroup 2.7 of FGE.19 (see Table 4). Twenty-three of the substances are α,β-unsaturated alicyclic ketones [FL-nos: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] and three are precursors for such ketones [FL-nos: 02.106, 09.305 and 09.525].

For 11 substances [FL-nos: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168] the Panel have ruled out concerns regarding genotoxicity in FGE.213.

In FGE.213Rev1, new data have been evaluated for the representative of the remaining substances. More specifically, data for β-ionone [FL-no: 07.008], β-damascone [FL-no: 07.083], maltol [FL-no: 07.014], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]. All these studies are fully compliant with current guidelines, and stand in contrast to earlier studies previously evaluated in FGE.213.

The combined evidence from *in vitro* and *in vivo* genotoxicity data for the selected representative substances β-ionone [FL-no: 07.008], β-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] does not indicate a genotoxic potential. Therefore, these substances and the nine substances that they represent [FL-nos: 02.106, 07.010, 07.041, 07.108, 07.127, 07.136, 07.200, 07.224 and 09.305] could be evaluated through the Procedure.

For maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525], the Panel concluded that the concern for genotoxicity could not be ruled out.

3. Assessment

3.1. Additional data evaluated by the Panel in FGE.213Rev2

3.1.1. Plasma bioanalysis

Since data provided to prove the systemic exposure to maltol of the animals tested in the combined bone marrow micronucleus test and comet assay in liver (Beevers, 2013a) were considered inconclusive in FGE.213Rev1, the Flavour Industry has submitted a new plasma bioanalysis (Beevers, 2015).

Six male Han Wistar rats were dosed at 700 mg maltol/kg bw/day (determined previously as an estimate of the MTD), using the same dosing regimen employed in the combined micronucleus test and comet assay (Beevers, 2013a).

Maltol was prepared as a suspension 0.5 % (w/v) in aqueous methylcellulose and administered via oral gavage at: 0 (day 1), 24 (day 2) and 45 (day 3) hours. Whole blood was collected at 0.5, 1, 2 and 3 hours after dosing on day 3. Plasma was isolated and analysed using GC–MSD. Ethyl maltol was used as an internal standard.

Detectable levels of maltol were found in all plasma samples isolated at 0.5, 1 and 2 hours after dosing. Peak plasma levels of maltol were seen in the majority of animals at 0.5 hours after dose administration. The concentration of maltol detected in plasma was different between the animals of 2 separate cages (treated with the same dose) and the authors of the study did not identify any technical reasons that could account for this difference. However, it was concluded that data demonstrate the presence of maltol in blood and that accordingly the bone marrow could be considered exposed.

3.1.2. Additional *in vitro* data on maltol

An *in vitro* chromosomal aberration assay on maltol (not available before) is considered in the present revision of FGE.213. Maltol (purity 99 %) was tested in CHL fibroblast cell line at three concentrations: 25, 50 and 75 µg/ml. Cells were harvested for chromosomal preparations after 24 hours or 48 hours from the beginning of the treatment; metabolic activation was not included. Structural chromosomal aberrations were observed at the 2 highest concentrations tested after both 24 hours and 48 hours of treatment. These increases were concentration related, polyploidy was not observed (Ishidate, 1988). The result obtained in this study is consistent with the clastogenic effect of maltol observed in the study by Whitwell (2012), described in Section 2.5.2.

3.1.3. Additional information on *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127]

In FGE.213Rev1, the Panel concluded that the genotoxicity concern for *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] could be ruled out based on available genotoxicity data on structurally related substances and consequently could be evaluated through the Procedure in FGE.57Rev1.

During the evaluation of *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] through the Procedure, the Panel noted that the chemical structure of *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] is actually more closely related to the structure of pulegone than to the structures used for the read-across approach in FGE.213Rev1.

New information was found on genotoxicity and carcinogenicity of pulegone, based on which additional data are expected to be provided by the applicant. At present, the data available on pulegone and on the structurally related substance *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] do not rule out the concern for genotoxicity and carcinogenicity. Therefore *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] will be re-evaluated pending the submission of additional genotoxicity data.

4. Conclusion

The Panel considered the available genotoxicity studies on maltol. An *in vivo* study in mice (by intraperitoneal route) showed that maltol induced a statistically significant increase in the incidence of micronuclei in bone marrow cells. Since maltol is intended to be used as food flavouring agent, the *in vivo* study performed by gavage (combined micronucleus test and comet assay in rats) was considered more relevant, but in the previous revision of that opinion, this study did not allow conclusions on the genotoxicity of maltol to be made because exposure of target tissue was not demonstrated. Therefore, the Panel requested to investigate the systemic exposure of animals to maltol.

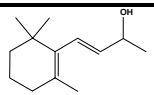
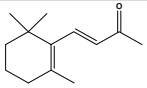
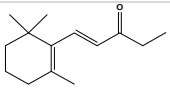
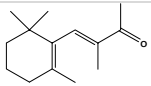
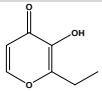
The Panel considered the new plasma bioanalysis for maltol and concluded that, based on the new data provided, it now seems justifiable to assume that the animals were systemically exposed to maltol and that the bone marrow was exposed in the *in vivo* micronucleus assay.

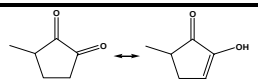
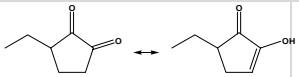
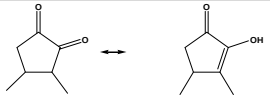
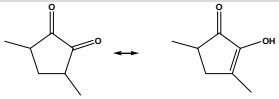
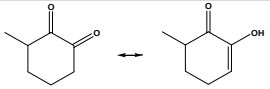
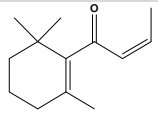
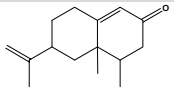
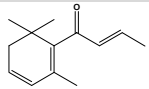
Therefore, the negative result of the *in vivo* micronucleus assay can be considered reliable and, accordingly, the concern for genotoxicity for maltol [FL-no: 07.014] and for maltyl isobutyrate [FL-no: 09.525] in food is ruled out; both substances were evaluated by JECFA before 2000 and no EFSA consideration is required.

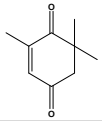
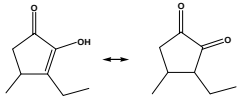
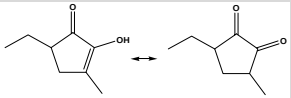
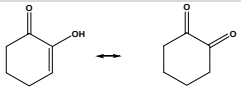
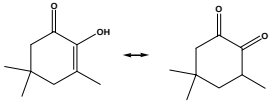
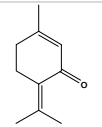
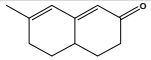
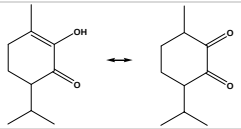
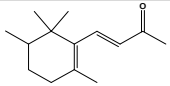
The Panel reconsidered the available data on *p*-mentha-1,4(8)-dien-3-one [FL-no: 07.127] based on new data for the structurally related substance pulegone, and concluded that additional genotoxicity data are needed for [FL-no: 07.127] to rule out the concern for genotoxicity.

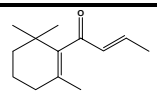
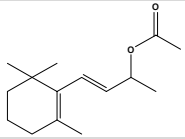
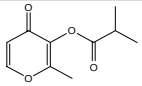
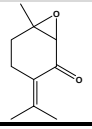
4.1. Summary of Specification for Substances in FGE.213 (JECFA 1998, 2000, 2005a, b, 2009b)

Table 4: Specification summary of the substances in FGE.213

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
02.106 **392**	4-(2,2,6-Trimethyl-1-cyclohexenyl)but-3-en-2-ol		3625 – 22029-76-1	Liquid $C_{13}H_{22}O$ 194.32	– –	107 (4 hPa) – IR 92 %	1.499 0.927–0.933
07.008 **389**	β-Ionone		2595 142 14901-07-6	Liquid $C_{13}H_{20}O$ 192.30	Insoluble 1 ml in 3 ml 70 % alcohol	239 – IR 95 %	1.517–1.522 0.940–0.947
07.010 **399**	Methyl-β-ionone		2712 144 127-43-5	Liquid $C_{14}H_{22}O$ 206.33	– –	238–242 – IR 88 %	1.503–1.508 0.930–0.935
07.014 **1480**	Maltol		2656 148 118-71-8	Solid $C_6H_6O_3$ 126.11	Very slightly soluble Soluble	– 159–162 NMR 98 %	n.a. n.a.
07.041	β-Isomethylionone		4151 650 79-89-0	Solid $C_{14}H_{22}O$ 206.32	– Freely soluble	334 62 – 95 %	n.a. n.a.
07.047 **1481**	Ethyl maltol		3487 692 4940-11-8	Solid $C_7H_8O_3$ 140.14	Soluble Soluble	– 89–93 NMR 99 %	n.a. n.a.

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
07.056 **418**	3-Methylcyclopentan-1,2-dione		2700 758 80-71-7	Solid $C_6H_8O_2$ 112.13	1 g in 72 ml water 1 g in 5 ml 90 % alcohol	– 104–108 IR 95 %	– –
07.057 **419**	3-Ethylcyclopentan-1,2-dione		3152 759 21835-01-8	Solid $C_7H_{10}O_2$ 126.16	Miscible –	78–80 (5 hPa) 36–43 IR 90 %	1.47–1.48 (25°) 1.060–1.066
07.075 **420**	3,4-Dimethylcyclopentan-1,2-dione		3268 2234 13494-06-9	Solid $C_7H_{10}O_2$ 126.16	– –	66 (1 hPa) 68–72 IR 98 %	– –
07.076 **421**	3,5-Dimethylcyclopentan-1,2-dione		3269 2235 13494-07-0	Solid $C_7H_{10}O_2$ 126.16	Insoluble –	– 87–93 MS 98 %	– –
07.080 **425**	3-Methylcyclohexan-1,2-dione		3305 2311 3008-43-3	Solid $C_7H_{10}O_2$ 126.16	Insoluble –	69–72 (1 hPa) 57–63 IR 98 %	– –
07.083 **384**	β-Damascone		3243 2340 23726-92-3	Liquid $C_{13}H_{20}O$ 192.30	– 1 ml in 10 ml 95 %	67–70 – IR 90 %	1.496–1.501 0.934–0.942 (20°)
07.089 **1398**	Nootkatone		3166 11164 4674-50-4	Liquid $C_{15}H_{22}O$ 218.35	Slightly soluble Soluble	73–103 (1 hPa) – NMR 93 %	1.510–1.523 1.003–1.032
07.108 **387**	β-Damascenone		3420 11197 23696-85-7	Liquid $C_{13}H_{18}O$ 190.28	– 1 ml in 10 ml 95 % alcohol	60 – IR 98 %	1.508–1.514 0.945–0.952 (20°)

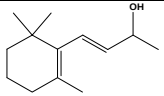
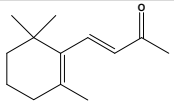
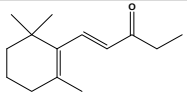
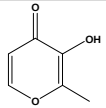
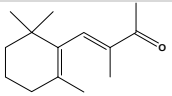
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
07.109 **1857**	2,6,6-Trimethylcyclohex-2-en-1,4-dione		3421 11200 1125-21-9	Solid $C_9H_{12}O_2$ 152.2	Slightly soluble Soluble	222 23–28 IR NMR 98 %	n.a. n.a.
07.117 **422**	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		3453 11077 42348-12-9	Liquid $C_8H_{12}O_2$ 140.18	Slightly insoluble Miscible	– – NMR 99 %	1.481–1.487 1.055–1.061
07.118 **423**	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		3454 11078 53263-58-4	Liquid $C_8H_{12}O_2$ 140.18	Slightly soluble Soluble	– – NMR 99 %	1.478–1.484 1.053–1.060
07.119 **424**	2-Hydroxycyclohex-2-en-1-one		3458 11046 10316-66-2	Solid $C_6H_8O_2$ 112.13	Soluble Soluble	53 (3 hPa) 35–38 IR 99.3 %	– –
07.120 **426**	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		3459 11198 4883-60-7	Solid $C_9H_{14}O_2$ 154.21	Slightly soluble Soluble	90–100 (20 hPa) 88 IR 99 %	– –
07.127 **757**	*p*-Mentha-1,4(8)-dien-3-one		3560 11189 491-09-8	Liquid $C_{10}H_{14}O$ 150.22	Insoluble Miscible	233 – MS 95 %	1.472–1.478 0.976–0.983
07.136 **1405**	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		3715 – 34545-88-5	Solid $C_{11}H_{14}O$ 162.23	Insoluble Soluble	n.a. 36–37 IR 99 %	n.a. n.a.
07.168 **2038**	2-Hydroxypiperitone		4143 – 490-03-9	Solid $C_{10}H_{16}O_2$ 168.24	Slightly soluble Freely soluble	233 82 NMR MS 98 %	n.a. n.a.
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		– – 79-70-9	Liquid $C_{14}H_{22}O$ 206.33	Practically insoluble or insoluble Freely soluble	108 (2 hPa) – MS 95 %	1.515–1.521 0.943–0.949

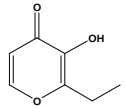
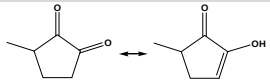
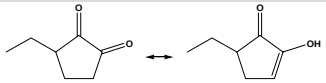
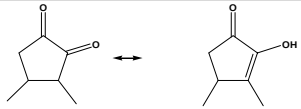
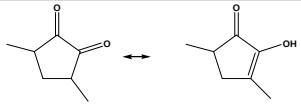
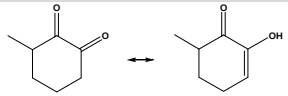
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
07.224	tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one		3243 2340 23726-91-2	– – –	– –	– – – 90 %	– –
09.305 **1409**	β-Ionyl acetate		3844 10702 22030-19-9	Liquid $C_{15}H_{24}O_2$ 236.35	Insoluble Soluble	120 (3 hPa) – NMR 92 %	1.474–1.484 0.934–0.944
09.525 **1482**	Maltyl isobutyrate		3462 10739 65416-14-0	Liquid $C_{10}H_{12}O_4$ 196.20	Insoluble Soluble	100 (0.01 hPa) – IR 96 %	1.493–1.501 1.140–1.153
16.044 **1574**	Piperitenone oxide		4199 10508 35178-55-3	Solid $C_{10}H_{14}O_2$ 166.22	Soluble Soluble	– 25 NMR MS 95 %	n.a. n.a.

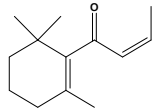
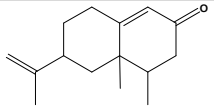
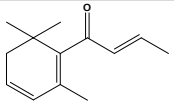
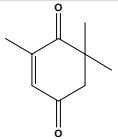
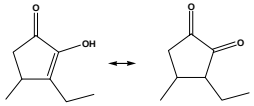
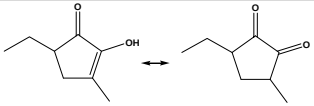
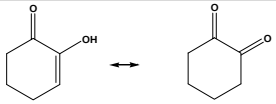
n.a.: not applicabile; (-): data not reported
(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95 % ethanol, if not otherwise stated.
(c): At 1 013.25 hPa, if not otherwise stated.
(d): At 20 °C, if not otherwise stated.
(e): At 25 °C, if not otherwise stated.

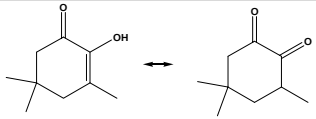
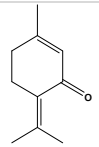
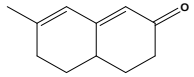
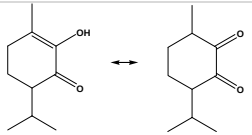
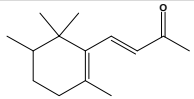
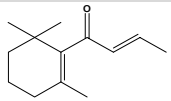
4.2. Summary of Safety Evaluation Applying the Procedure (JECFA 1999, 2001, 2006a, b, 2009a)

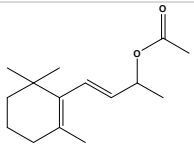
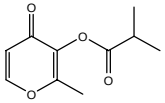
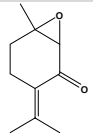
Table 5: Summary of safety evaluation applying the procedure

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [a] US MSDI (μg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
02.106 **392**	4-(2,2,6-Trimethyl-1-cyclohexenyl)but-3-en-2-ol		0.73 0.1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.008 **389**	β-Ionone		130 100	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.010 **399**	Methyl-β-ionone		5.4 0.2	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.014 **1480**	Maltol		3 060 2 898	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev2, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.041	β-Isomethylionone		0.011	Not evaluated by the JECFA		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.12Rev5.

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [a] US MSDI (μg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
07.047 **1481**	Ethyl maltol		1 580 6 692	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.83Rev1. No safety concern at the estimated level of intake based on the MSDI approach.
07.056 **418**	3-Methylcyclopentan-1,2-dione		570 710	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.057 **419**	3-Ethylcyclopentan-1,2-dione		32 23	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.075 **420**	3,4-Dimethylcyclopentan-1,2-dione		30 2	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.076 **421**	3,5-Dimethylcyclopentan-1,2-dione		35 29	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.080 **425**	3-Methylcyclohexan-1,2-dione		1.3 8	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [a] US MSDI (μg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
07.083 **384**	β-Damascone		37 10	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.089 **1398**	Nootkatone		130 20	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.87Rev2.
07.108 **387**	β-Damascenone		73 5	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.109 **1857**	2,6,6-Trimethylcyclohex-2-en-1,4-dione		50	Class II No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.09Rev5.
07.117 **422**	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		ND 0.17	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.118 **423**	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		ND 0.38	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.119 **424**	2-Hydroxycyclohex-2-en-1-one		0.049 0.76	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA

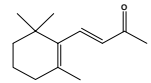
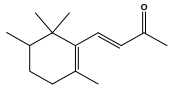
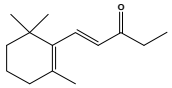
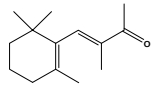
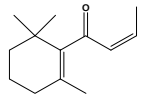
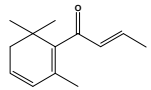
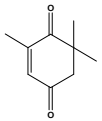
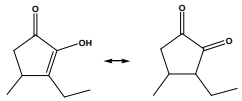
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [a] US MSDI (μg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound [d] or [e]	EFSA conclusion on the named compound (genotoxicity)
						before 2000. No EFSA consideration required.
07.120 **426**	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		1.2 2	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.127 **757**	*p*-Mentha-1,4(8)-dien-3-one		0.012 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1 and FGE.213Rev2, genotoxicity concern could not be ruled out. Additional data are requested.
07.136 **1405**	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		ND 0.04	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.87Rev2.
07.168 **2038**	2-Hydroxypiperitone		0.0012	Class III A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.11Rev2. No safety concern at the estimated level of intake based on the MSDI approach.
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		0.012	Class I No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.12Rev5.
07.224	tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one		100	No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated

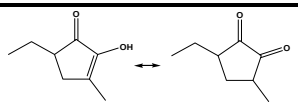
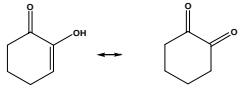
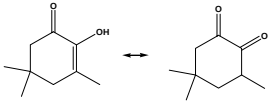
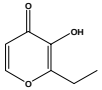
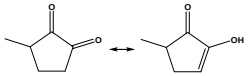
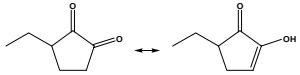
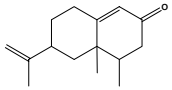
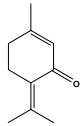
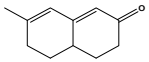
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [a] US MSDI (μg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
						using the Procedure in FGE.12Rev5.
09.305 **1409**	β-Ionyl acetate		ND 9	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.73Rev3. MSDI based on USA production figure.
09.525 **1482**	Maltyl isobutyrate		20 38	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev2, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
16.044 **1574**	Piperitenone oxide		0.012 0.2	Class III A3: Intake below threshold	(d)	Evaluated in FGE.213, additional genotoxicity data required. The substance is not supported by the Flavour Industry any longer. No further evaluation.

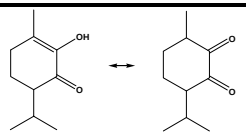
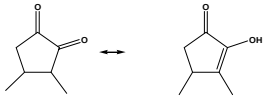
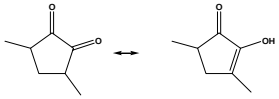
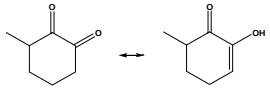
ND: no data
(a): EU MSDI: Amount added to food as flavour in (kg/year) x 10E9/(0.1 × population in Europe (= 375 × 10E6) × 0.6 × 365) = μg/capita/day
(b): Thresholds of concern: class I = 1800 μg/person/day, class II = 540 μg/person/day, Class III = 90 μg/person/day
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot
(d): No safety concern based on intake calculated by the MSDI approach of the named compound
(e): Data must be available on the substance or closely related substances to perform a safety evaluation

4.3. (Q)SAR Predictions on Mutagenicity in Five Models for 22 Ketones from Subgroup 2.7

Table 6: (Q)SAR predictions on mutagenicity for 22 alicyclic ketones from subgroup 2.7

FL-no JECFA-no	EU Register name	Structural formula [a]	ISS Local Model Ames Test TA100 [b]	MultiCASE Ames test [c]	MultiCASE Mouse lymphoma test [d]	MultiCASE Chromosomal aberration test in CHO [e]	MultiCASE Chromosomal aberration test in CHL [f]
07.008 389	β-Ionone		NEG	NEG	NEG	NEG	EQU
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		NEG	NEG	NEG	NEG	EQU
07.010 399	Methyl-β-ionone		NEG	NEG	OD	OD	EQU
07.041	β-Isomethylionone		NEG	EQU	NEG	NEG	NEG
07.083 384	β-Damascone		OD	NEG	OD	OD	EQU
07.108 387	β-Damascenone		OD	NEG	OD	OD	EQU
07.109	2,6,6-Trimethylcyclohex-2-en-1,4-dione		OD	NEG	OD	NEG	EQU
07.117 422	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		OD	NEG	NEG	OD	NEG

FL-no JECFA-no	EU Register name	Structural formula [a]	ISS Local Model Ames Test TA100 [b]	MultiCASE Ames test [c]	MultiCASE Mouse lymphoma test [d]	MultiCASE Chromosomal aberration test in CHO [e]	MultiCASE Chromosomal aberration test in CHL [f]
07.118 423	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		OD	NEG	NEG	NEG	NEG
07.119 424	2-Hydroxycyclohex-2-en-1-one		OD	OD	NEG	OD	NEG
07.120 426	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		OD	NEG	NEG	OD	NEG
07.014 1480	Maltol		OD	OD	NEG	OD	POS
07.047 1481	Ethyl maltol		OD	OD	NEG	OD	POS
07.056 418	3-Methylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG
07.057 419	3-Ethylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG
07.089 1398	Nootkatone		OD	NEG	NEG	NEG	POS
07.127 757	*p*-Mentha-1,4(8)-dien-3-one		OD	NEG	OD	NEG	NEG
07.136 1405	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		OD	NEG	NEG	NEG	OD

FL-no JECFA-no	EU Register name	Structural formula [a]	ISS Local Model Ames Test TA100 [b]	MultiCASE Ames test [c]	MultiCASE Mouse lymphoma test [d]	MultiCASE Chromosomal aberration test in CHO [e]	MultiCASE Chromosomal aberration test in CHL [f]
07.168 -	2-Hydroxypiperitone		OD	NEG	NEG	NEG	NEG
07.075 420	3,4-Dimethylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG
07.076 421	3,5-Dimethylcyclopentan-1,2-dione		OD	NEG	NEG	NEG	NEG
07.080 425	3-Methylcyclohexan-1,2-dione		OD	NEG	NEG	OD	NEG

OD, out of applicability domain: not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physicochemical, structural, biological etc.
(a): Structure group 2.7: α,β-unsaturated ketones.
(b): Local model on aldehydes and ketones, Ames TA100. (NEG: Negative; POS: Positive; OD*: out of domain).
(c): MultiCASE Ames test (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(d): MultiCASE Mouse Lymphona test (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(e): MultiCASE Chromosomal aberration in CHO (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(f): MultiCASE Chromosomal aberration in CHL (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

4.4. Genotoxicity data (*in vitro*) considered by the Panel in FGE.213

Table 7: Genotoxicity (*in vitro*)

Chemical name [FL-no]	Test system	Test object	Concentration	Reported result	Reference	Comments [(d)]
β-Ionone [07.008]	Gene mutation (preincubation)	*S. typhimurium* TA98, TA100, TA1535, TA1537	1–180 µg/plate	Negative [(a)]	Mortelmans et al., 1986	Valid.
	Gene mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	3 mmol/plate	Negative [(a)]	Florin et al., 1980	Insufficient validity (spot test, not according to OECD guideline, methods and results insufficiently reported).
3-Methylcyclopentan-1,2-dione [07.056]	Reverse mutation	*S. typhimurium* TA1535	10 000 µg/plate	Negative [(b)]	Heck et al., 1989	Validity cannot be evaluated (result not reported in detail).
	Unscheduled DNA synthesis	Rat hepatocytes	500 µg/plate	Negative [(b)]	Heck et al., 1989	Validity cannot be evaluated (result not reported in detail).
Maltol [07.014]	Reverse mutation	*S. typhimurium* TA100	4.44 µmol/plate (560 µg/plate)	Negative [(c)]	Kim et al., 1987	Insufficient validity (only one concentration was tested with only one bacterial strain without metabolic activation). The main purpose of the study was to investigate antimutagenic effects.
	Reverse mutation	*S. typhimurium* TA98 and TA100	Up to 3 mg/plate (3 000 µg/plate)	Positive [(a)]	Bjeldanes and Chew, 1979	Valid.
	Reverse mutation	*S. typhimurium* TA92, TA98, TA100 and TA104	1.5 to 11 µmol/plate (189 to 1 387 µg/plate)	Negative	Gava et al., 1989	Limited validity (data not reported in detail).
	Reverse mutation	*S. typhimurium* TA1535, TA98, TA100 and TA1537	33 to 10 000 µg/plate	Positive [(b)]	Mortelmans et al., 1986	Valid.
	Reverse mutation	*S. typhimurium* TA97 and TA102	0.1, 0.5, 1, 5, or 10 mg/plate (100, 500, 1 000, 5 000, or 10 000 µg/plate)	Weak Positive [(a)]	Fujita et al., 1992	Result is considered equivocal. Limited validity (the use of only two strains is not according to OECD guideline).
	DNA damage (SOS Chromotest)	*Escherichia coli* PQ37	5 mM (631 µg/ml)	Negative	Ohshima et al., 1989	The test system used is considered inappropriate, due to insufficient validity.

Chemical name [FL-no]	Test system	Test object	Concentration	Reported result	Reference	Comments [(d)]
	Sister chromatid exchange	Chinese hamster ovary cells	Up to 1.5 μmol/ml (12.6 to 189 μg/ml)	Positive [(c)]	Gava et al., 1989	Validity cannot be evaluated (insufficiently reported: number of cells analysed not reported. Statistical test used not reported). SCEs were reported as SCE per chromosome. Effect was less than twofold compared to control.
	Sister chromatid exchange	Human lymphocytes	Up to 1.0 mM (126.11 μg/ml)	Positive	Jansson et al., 1986	Validity cannot be evaluated. Relevance of test system for the evaluation of genotoxicity uncertain.
Ethyl maltol [07.047]	Reverse mutation	*S. typhimurium* TA 1535, TA1537, TA1538, TA98 and TA100	5 concentrations up to cytotoxicity, or max. 3 600 μg/plate	Negative [(a)]	Wild et al., 1983	Limited validity (result not reported in details, no TA102 or E. Coli).
	Reverse mutation	*S. typhimurium* TA98 and TA100	Up to 2 mg/plate (2 000 μg/plate)	Positive [(a)]	Bjeldanes and Chew, 1979	Valid.

(a): With and without metabolic activation
(b): With metabolic activation
(c): Without metabolic activation
(d): Validity of genotoxicity studies:

- Valid
- Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and/or limited documentation)
- Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system)
- Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

4.5. Genotoxicity data (*in vivo*) considered by the Panel in FGE.213

Table 8: Genotoxicity (*in vivo*)

Chemical name [FL-no]	Test system	Test object	Route	Dose	Result	Reference	Comments [(a)]
Maltol [07.014]	Micronucleus formation	ddY Mouse bone marrow cells	Intraperitoneal	125, 250, or 500 mg/kg	Positive	Hayashi et al., 1988	Valid. The induction of micronuclei was up to about 10-fold compared with control
	Sex-linked Recessive Lethal Mutation	*Drosophila melanogaster*	Feed	6 000 ppm (6000 µg/ml)	Equivocal	Zimmering et al., 1989	Limited validity (only one exposure level tested). Test system considered of limited relevance.
	Sex-linked recessive lethal mutation	*Drosophila melanogaster*	Feed	10 000 ppm (10 000 µg/ml)	Negative	Mason et al., 1992	Valid, however, test system considered of limited relevance.
	Sex-linked recessive lethal mutation	*Drosophila melanogaster*	Injection	0.2 – 0.3 µl, 10 000 ppm (10 000 µg/ml)	Negative	Mason et al., 1992	Valid, however, test system considered of limited relevance.
Ethyl maltol [07.047]	Micronucleus formation	NMRI Mouse bone marrow cells	Intraperitoneal	420, 700, or 980 mg/kg	Negative	Wild et al., 1983	Limited validity (injected twice; only analysis at one time point; no PCE/NCE ratio reported).
	Micronucleus formation	NMRI mouse bone marrow cells	Intraperitoneal	980 mg/kg	Negative	Wild et al., 1983	Limited validity (single injection, analysis at three time points, no PCE/NCE ratio reported).
	Sex-linked recessive lethal mutation (Basc test)	*Drosophila melanogaster*	Feed	14, 25 or 50 mM	Negative	Wild et al., 1983	Limited validity (limited reporting, test system considered of limited relevance).

(a): Validity of genotoxicity studies:

- Valid
- Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and/or limited documentation)
- Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system)
- Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too few experimental details provided)

4.6. Carcinogenicity studies considered by the Panel in FGE.213

Table 9: Carcinogenicity studies

Chemical name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	Results	Reference	Comments [(a)]
Ethyl maltol [07.047]	Rats; Male, Female 25/sex/group	Diet	0, 50, 100 and 200 mg/kg bw/day	2 years	Males: No increase in tumour incidences Females: No increase in tumour incidences	Gralla et al., 1969	Valid. The study was performed before the introduction of OECD guidelines but is, however, considered valid. The NOAEL was 200 mg/kg bw/day, the highest dose tested
3-Ethylcyclopentan-1,2-dione [07.057]	Rats; Male, Female 50/sex/group	Diet	0, 30, 80 and 200 mg/kg bw/day	2 years	Males: No increase in tumour incidences Females: No increase in tumour incidences	King et al., 1979	Valid. The study was performed before the introduction of OECD guidelines but is, however, considered valid. The NOAEL was 200 mg/kg bw/day, the highest dose tested

(a): Validity of genotoxicity studies:

- Valid
- Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and/or limited documentation)
- Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system)
- Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too few experimental details provided)

4.7. Genotoxicity data (*in vitro*) considered by the Panel in FGE.213Rev1 and FGE.213Rev2

Table 10: Summary of additional *in vitro* genotoxicity data for FGE.213Rev1

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
β-Ionone [07.008]	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.32–5000 µg/plate (a, b)	Negative	Ballantyne, 2011	Evidence of toxicity was observed in all strains at concentrations of 1000 µg/plate and greater in the absence and in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA98, TA100, TA102, TA1535 and TA1537	10.24–1000 µg/plate (b, d) or (c, e)	Negative		Evidence of toxicity was observed in all strains at 1 000 µg/plate in the absence and presence of S9-mix, and in most cases these toxic effects also extended down to concentrations of 160 or 400 µg/plate. Study design complied with current recommendations.
	Micronucleus assay	Human peripheral blood lymphocytes	30–60 µg/ml (d, f) 80–120 µg/ml (e, f) 5–17.5 µg/ml (d, g)	Negative	Stone, 2011a	The top concentrations induced 50–60 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
Maltol [07.014]	Reverse mutation	*S. typhimurium* TA98, TA100 and TA102, TA1535 and TA1537	0.32–5000 µg/plate [a, b]	Negative	Ballantyne, 2012	Evidence of toxicity was observed in TA102 at concentrations of 1000 and 5000 µg/plate in the absence of S9-mix and at concentrations of 200 µg/plate and greater in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA98, TA100, TA1535 and TA1537	51.2–5000 µg/plate (b, d) or (c, e)	Negative		Toxicity was observed at 5000 µg/plate in strain TA100 only in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA102	20.48–5000 µg/plate (b, d) or (c, e)	Negative		Evidence of toxicity was observed at 5 000 µg/plate in the absence and presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus assay	Human peripheral blood lymphocytes	400–1262 µg/ml [d, f] 400–1262 µg/ml [e, f] 125–300 µg/ml [d, g]	Equivocal Positive Negative	Whitwell, 2012	The top concentrations in the 3 + 21 hours treatments in the absence and presence of S9-mix induced, respectively, 24 % and 19 % of toxicity. The top concentration in the 24 + 0 hours treatment in the absence of S9-mix induced 57 % toxicity. There was evidence of micronuclei induction when tested for 3 + 21 hours in the presence of S9-mix, while in absence of S9-mix the data were considered equivocal. However, no induction of micronuclei was observed in the continuous exposure test. Study design complies with OECD Guideline 487.
	Chromosomal aberration test	CHL cells	25, 50, 75 µg/ml (d, g) or (d, h)	Positive	Ishidate, 1988	Structural chromosomal aberrations were observed at the 2 highest concentrations tested.

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
β-Damascone [07.083]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	0.32–5000 µg/plate (a, b)	Negative	Bowen, 2011b	Toxicity was observed at 1 000 and/or 5 000 µg/plate across all strains in the absence and presence of S9-mix; no clear evidence of toxicity in TA100 in the presence of S9-mix. No statistically significant increase in revertant numbers was seen at any concentration, either in the presence or absence of S9-mix.
		S. typhimurium, TA1535, TA1537 and TA102 *S. typhimurium* TA98, TA100	78.13–2500 µg/plate (b, d) or (c, e) 156.3–5000 µg/plate (b, d) or (c, e)	Negative Negative		Evidence of toxicity was observed at the highest three or four concentrations across all strains in the absence and presence of S9-mix. No statistically significant increase in revertant numbers was seen at any concentration, either in the presence or absence of S9-mix
		S. typhimurium TA98	19.3–1250 µg/plate (c, e)	Negative		Evidence of toxicity was observed at the highest four concentrations in strain TA98 in the presence of S9-mix. No statistically significant increase in revertant numbers was seen at any concentration, in the presence of S9-mix.
	Micronucleus assay	Human peripheral blood lymphocytes	8–22 µg/ml (d, f) 12–18 µg/ml (e, f) 6–9 µg/ml (d, g)	Equivocal(d, f) Positive (e, f) Equivocal(d, g)	Stone, 2012	Positive result was obtained in the 3 + 21 hour treatment in the presence of S9-mix. Study design complies with OECD Guideline 487.
Nootkatone [07.089]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	0.5–50 µg/plate (b, d) 1.5.5–150 µg/plate (b, e) 0.5–50 µg/plate (b, d) 0.5–150 µg/plate (c, e)	Negative	Marzin, 1998	Evidence of toxicity was observed at 50 µg/plate in all strains in the absence of S9-mix and at 150 µg/plate in all strains in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus assay	Human peripheral blood lymphocytes	50–80 µg/ml (d, f) 160–185 µg/ml (e, f) 10–24 µg/ml (d, g)	Negative	Stone, 2011b	The top concentrations in all parts of the study induced >50 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.

Chemical name [FL-no]	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
2,6,6-Trimethylcyclohex-2-en-1,4-dione [07.109]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	0.32–5000 µg/plate (a, b)	Negative	Bowen, 2011a	Evidence of toxicity was observed at 1000 and/or 5000 µg/plate in strains TA102 and TA1535 in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	156.3–5000 µg/plate (b, d) or (c, e)	Negative		Evidence of toxicity was observed in TA102 at 2500 and 5000 µg/plate. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus Assay	Human peripheral blood lymphocytes	500–1522 µg/ml (d, f) 1000–1522 µg/ml (e, f) 300–550 µg/ml (d, g)	Negative	Lloyd, 2011	The top concentrations in the 3 + 21 hours in the absence and presence of S9-mix were 10 mM. The top concentration in the 24 + 0 hours in the absence of S9-mix induced 57 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.

(a): With and without S9-mix metabolic activation
(b): Plate incorporation method
(c): Without S9-mix metabolic activation
(d): Pre-incubation method
(e): With S9-mix metabolic activation
(f): 3-hour incubation with a 21-hour recovery period
(g): 24-hour incubation with no recovery period
(h): 48-hour incubation with no recovery period

4.8. Genotoxicity data (*in vivo*) considered by the Panel in FGE.213Rev1 and FGE.213Rev2

Table 11: Summary of additional *in vivo* genotoxicity data submitted for FGE.213Rev1 and FGE.213Rev2

Chemical name [FL-no]	Test system	Test object	Route	Dose	Result	Reference	Comments
Maltol [07.014]	Micronucleus assay	Han Wistar Rat; M	Gavage	70, 350, 700 mg/kg bw/day [(a)]	Negative	Beevers, 2013a	The average MNPCE appearance frequency and ratio of PCE at all dose levels fell within concurrent and historical control ranges. However, evidence of target tissue exposure was inconclusive. The study was performed in compliance with OECD Guideline 474. A further plasma analysis was performed (Beevers, 2015) showing the systemic exposure of animals to maltol. Based on the new bioanalysis, results of the micronucleus assay were considered as negative.
	Comet assay	Han Wistar rat; M	Gavage		Negative		Mean percentage tail intensity and mean tail moment were within historical control range at all test doses. The study was performed in compliance with recommendations of the comet and IWGT workshop, Japanese Centre for the Validation of Alternative Methods (JaCVAM) and current literature.
β-Damascone [07.083]	Micronucleus assay	Han Wistar rat; M	Gavage	125, 250 and 500 mg/kg bw/day [(a)]	Negative	Beevers, 2013b,c	The average MNPCE appearance frequency and ratio of PCE at all dose levels fell within concurrent and historical control ranges. The study was performed in compliance with OECD Guideline 474.
	Comet assay	Han Wistar rat; M	Gavage		Negative		Mean% tail intensity and mean tail moment were within historical control range at all test doses. The study was performed in compliance with recommendations of the comet and IWGT workshop, Japanese Centre for the Validation of Alternative Methods (JaCVAM) and current literature.

(a): Administered via gavage in 3 doses at times 0, 24 and 45 hours with sacrifice and harvest at 48 hours

Documentation provided to EFSA

1. Ballantyne M, 2011. Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*. beta-Ionone. Covance Laboratories LTD. Study no. 8250470. October 2011. Unpublished report submitted by EFFA to FLAVIS Secretariat.

2. Ballantyne M, 2012. Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*. Maltol. Covance Laboratories LTD. Study no. 8250465. January 2012. Unpublished report submitted by EFFA to FLAVIS Secretariat.

3. Beevers C, 2013a. Combined bone marrow micronucleus test and comet assay in the liver of treated rats. Maltol. Covance Laboratories Ltd. Study no. 8262049. February 2013. Unpublished report submitted by EFFA to FLAVIS Secretariat.

4. Beevers C, 2013b. Draft report. Analysis of comet slides from Covance Study 8262048. beta-Damascone. Covance Laboratories Ltd. Study no. 8281500. April 2013. Unpublished report submitted by EFFA to FLAVIS Secretariat.

5. Beevers C, 2013c. Combined bone marrow micronucleus test and comet assay in the liver of treated rats. beta-Damascone. Covance Laboratories Ltd. Study no. 8262048. June 2013. Unpublished report submitted by EFFA to FLAVIS Secretariat.

6. Beevers C, 2015. Maltol: Bioanalysis investigation to support Covance study 8262049. Submitted by EFFA

7. Benigni R and Netzeva T, 2007a. Report on a QSAR model for prediction of genotoxicity of α,β-unsaturated aldehydes in S. typhimurium TA100 and its application for predictions on α,β -unsaturated aldehydes in Flavouring Group Evaluation 19 (FGE.19). Unpublished report submitted by FLAVIS Secretariat to EFSA.

8. Benigni R and Netzeva T, 2007b. Report on a QSAR model for prediction of genotoxicity of α,β-unsaturated ketones in S. typhimurium TA100 and its application for predictions on α,β-unsaturated aldehydes in Flavouring Group Evaluation 19 (FGE.19). Unpublished report submitted by FLAVIS Secretariat to EFSA.

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10. Bowen R, 2011b. Reverse mutation in five histidine-requiring strains of Salmonella typhimurium. beta-Damascone. Covance Laboratories Ltd. Study no. 8240842. May 2011. Unpublished report submitted by EFFA to FLAVIS Secretariat.

11. Gry J, Beltoft V, Benigni R, Binderup M-L, Carere A, Engel K-H, Gürtler R, Jensen GE, Hulzebos E, Larsen JC, Mennes W, Netzeva T, Niemelä J, Nikolov N, Nørby KK and Wedebye EB, 2007. Description and validation of QSAR genotoxicity models for use in evaluation of flavouring substances in Flavouring Group Evaluation 19 (FGE.19) on 360 α,β-unsaturated aldehydes and ketones and precursors for these. Unpublished report submitted by FLAVIS Secretariat to EFSA.

12. IOFI (International Organization of the Flavor Industry), 2012. Flavouring Group Evaluation 213 Flavouring Substance (Flavouring Substances) of the Chemical Group 3 (Annex I of 1565/2000/EC) Alicyclic α,β-unsaturated aldehydes, ketones and related substances with the α,β-conjugation in the ring or in the side chain, Alicyclic ketones - more complex, Chemical Subgroup 2.7 of FGE.19. 4/12/2012. FLAVIS/8.170.

13. IOFI (International Organization of the Flavor Industry), 2013. Flavouring Group Evaluation 19 Subgroup 2.4/2.7, alpha-Damascone and beta-Damascone: 3 Flavouring Substances of the Chemical Group 3 (Annex I of 1565/2000/EC) Alicyclic α,β-unsaturated aldehydes, ketones and related substances with the α,β-conjugation in the ring or in the side chain. Alicyclic ketones (α,β-unsaturation in sidechain) Used as Flavouring Substances. 19/04–2013. FLAVIS/8.198.

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Abbreviations

bw	Body Weight
CAS	Chemical Abstracts Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHO	Chinese Hamster Ovary (cells)
CHL	Chinese Hamster Lung (cells)
CoE	Council of Europe
EC	European Commission
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FISH	Fluorescence In Situ Hybridisation
FLAVIS (FL)	Flavour Information System (database)
GC–MSD	gas chromatography–mass selective detection
GLP	Good Laboratory Practice
ID	Identity
IOFI	International Organization of the Flavor Industry
IR	Infrared Spectroscopy
IWGT	International Workshops on Genotoxicity Testing
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MN	Micronuclei
MNBN	MicroNucleated BiNucleate cells
MNPCE	Micronucleated Polychromatic Erythrocytes
MS	Mass Spectrometry
MSDI	Maximised Survey-derived Daily Intake
MTD	Maximum Tolerated Dose
NCE	NormoChromatic Erythrocytes
NMR	Nuclear Magnetic Resonance
No	Number
NOEL	No Observed Effect Level
NOAEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
PCE	Polychromatic Erythrocytes
PHA	Phytohaemagglutinin
(Q)SAR	(Quantitative) Structure Activity Relationship
SCE	Sister Chromatid Exchange
WHO	World Health Organization

SCIENTIFIC OPINION

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Safety and efficacy of secondary aliphatic saturated or unsaturated alcohols, ketones, ketals and esters with a second secondary or tertiary oxygenated functional group belonging to chemical group 10 when used as flavourings for all animal species

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Abstract

Following a request from the European Commission, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of 11 compounds belonging to chemical group 10. They are currently authorised as flavours in food. The FEEDAP Panel concludes that: diacetyl [07.052] is safe at the proposed maximum use level of 25 mg/kg complete feed for all target species, except piglets, chickens for fattening, laying hens and cats, for which the proposed normal use level of 5 mg/kg is safe; 3-hydroxybutan-2-one [07.051], 3-methylcyclo-pentan-1,2-dione [07.056], 3-ethylcyclopentan-1,2-dione [07.057], pentan-2,3-dione [07.060], 3,4-dimethylcyclopentan-1,2-dione [07.075], 3,5-dimethyl cyclopentan-1,2-dione [07.076], hexan-3,4-dione [07.077] and sec-butan-3-onyl acetate [09.186] are safe at the proposed maximum dose level of 5 mg/kg for all target species; 2,6,6-trimethylcyclohex-2-en-1,4-dione [07.109] and 3-methylnona-2,4-dione [07.184] are safe only at concentrations below the proposed use levels (0.5 mg/kg for cattle, salmonids and non-food producing animals, and 0.3 mg/kg for pigs and poultry). No safety concern would arise for the consumer from the use of these compounds up to the highest proposed level in feeds. Hazards for skin and eye contact and respiratory exposure are recognised for the majority of the compounds under application. Most are classified as irritating to the respiratory system. For 3-hydroxybutan-2-one [07.051], diacetyl [07.052], pentan-2,3-dione [07.060], hexan-3,4-dione [07.077], 2,6,6-trimethylcyclohex-2-en-1,4-dione [07.109], 3-methylnona-2,4-dione [07.184] and sec-butan-3-onyl acetate [09.186], the maximum proposed use levels are considered safe for the environment. For cyclopentanediones (3-methylcyclopentan-1,2-dione [07.056], 3-ethylcyclopentan-1.2-dione [07.057], 3,4 dimethylcyclopentan-1,2-dione [07.075] and 3,5-dimethylcyclopentan-1,2-dione [07.076]) usage at levels up to 0.5 mg/kg feed is unlikely to have an adverse effect on the terrestrial or freshwater environments. Because all the compounds under assessment are used in food as flavourings and their function in feed is essentially the same as that in food, no further demonstration of efficacy is necessary.

Keywords: sensory additives, aromatic ketones, secondary alcohols, related esters, safety, chemical group 10

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Question number: EFSA-Q-2010-00874

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7 and in addition, Article 10(2) of that Regulation also specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, within a maximum of 7 years after the entry into force of this Regulation.

The European Commission received a request from Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG)[2] for authorisation of 11 substances belonging to chemical group (CG) 10 (3-hydroxybutan-2-one, diacetyl, 3-methylcyclo-pentan-1,2-dione, 3-ethylcyclopentan-1,2-dione, pentan-2,3-dione, 3,4-dimethylcyclopentan-1,2-dione, 3,5-dimethylcyclopentan-1,2-dione, hexan-3,4-dione, 2,6,6-trimethylcyclohex-2-en-1,4-dione, 3-methylnona-2,4-dione and sec-butan-3-onyl acetate), when used as feed additives for all animal species (category: sensory additives; functional group: flavourings). CG 10 for flavouring substances is defined in Commission Regulation (EC) No 1565/2000[3] as 'secondary aliphatic saturated or unsaturated alcohols/ketones/ketals/esters with a second secondary or tertiary oxygenated functional group'.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive) and under Article 10(2) (re-evaluation of an authorised feed additive). During the course of the assessment, the applicant withdrew the application for the use of chemically defined flavourings in water for drinking.[4] EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 1 July 2010.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment, and on the efficacy of 3-hydroxybutan-2-one [The EU Flavour Information System (FLAVIS) Number 07.051], diacetyl [07.052], 3-methylcyclopentan-1,2-dione [07.056], 3-ethylcyclopentan-1,2-dione [07.057], pentan-2,3-dione [07.060], 3,4-dimethylcyclopentan-1, 2-dione [07.075], 3,5-dimethylcyclopentan-1,2-dione [07.076], hexan-3,4-dione [07.077], 2,6,6-trimethylcyclohex-2-en-1,4-dione [07.109], 3-methylnona-2,4-dione [07.184] and sec-butan-3-onyl acetate [09.186], when used under the proposed conditions of use (see Section 3.1.3).

1.2. Additional information

Nine of the 11 substances have been assessed by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA; WHO, 2000) and were considered safe for use in food. No acceptable daily intake (ADI) values were established. The two compounds not assessed were 2,6,6-trimethyl-cyclohex-2-en-1,4-dione [07.109] and 3-methylnona-2,4-dione [07.184].

Subsequently, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) assessed the compounds belonging to CG 10 and concluded that none of the 11 compounds under application gave rise to safety concerns when used as flavour in food (EFSA 2008a, 2009; EFSA CEF Panel, 2011, 2014a,b,c).

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

[2] On 13/03/2013, EFSA was informed by the applicant that FFAC EEIG was liquidated on 19/12/2012 and their rights as applicant were transferred to FEFANA asbl (EU Association of Specialty Feed Ingredients and their Mixtures). Avenue Louise 130A, Box 1, 1050 Brussels, Belgium.

[3] Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council. OJ L 180, 19.7.2000, p. 8.

[4] On 10 March 2016, EFSA was informed by the European Commission on the withdrawal of the application for re-authorisation of chemically defined flavourings - use in water.

All 11 compounds are all currently listed in the European Union database of flavouring substances[5] and in the European Union Register of Feed Additives, and thus authorised for use in food and feed in the European Union (EU), respectively. They have not been previously assessed by EFSA as feed additives.

Regulation (EC) No 429/2008[6] allows substances already approved for use in human food to be assessed with a more limited procedure than for other feed additives. However, the use of this procedure is always subject to the condition that food safety assessment is relevant to the use in feed.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[7] in support of the authorisation request for the use of the compounds belonging to CG 10 as feed additives. The technical dossier was prepared following the provisions of Article 7 of Regulation (EC) No 1831/2003, Regulation (EC) No 429/2008 and the applicable EFSA guidance documents.

The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has sought to use the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers and experts' knowledge, to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of flavourings of the 'secondary aliphatic saturated or unsaturated alcohols/ketones/ketals/esters with a second secondary or tertiary oxygenated functional group' in animal feed. The Executive Summary of the EURL report can be found in Annex A.[8]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of aromatic ketones, secondary alcohols and related esters is in line with the principles laid down in Regulation (EC) No 429/2008 and the relevant guidance documents: Guidance for the preparation of dossiers for sensory additives (EFSA FEEDAP Panel, 2012a), Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008b), Guidance for the preparation of dossiers for additives already authorised for use in food (EFSA FEEDAP Panel, 2012b), Guidance for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2012c), and Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012d).

3. Assessment

3.1. Characterisation

3.1.1. Characterisation of the flavouring additives

The molecular structures of the 11 additives under application are shown in Figure 1 and their physicochemical characteristics in Table 1.

[5] Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p. 1.

[6] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

[7] FEED dossier reference: FAD-2010-0026.

[8] The full report is available on the EURL website https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2010-0026.pdf

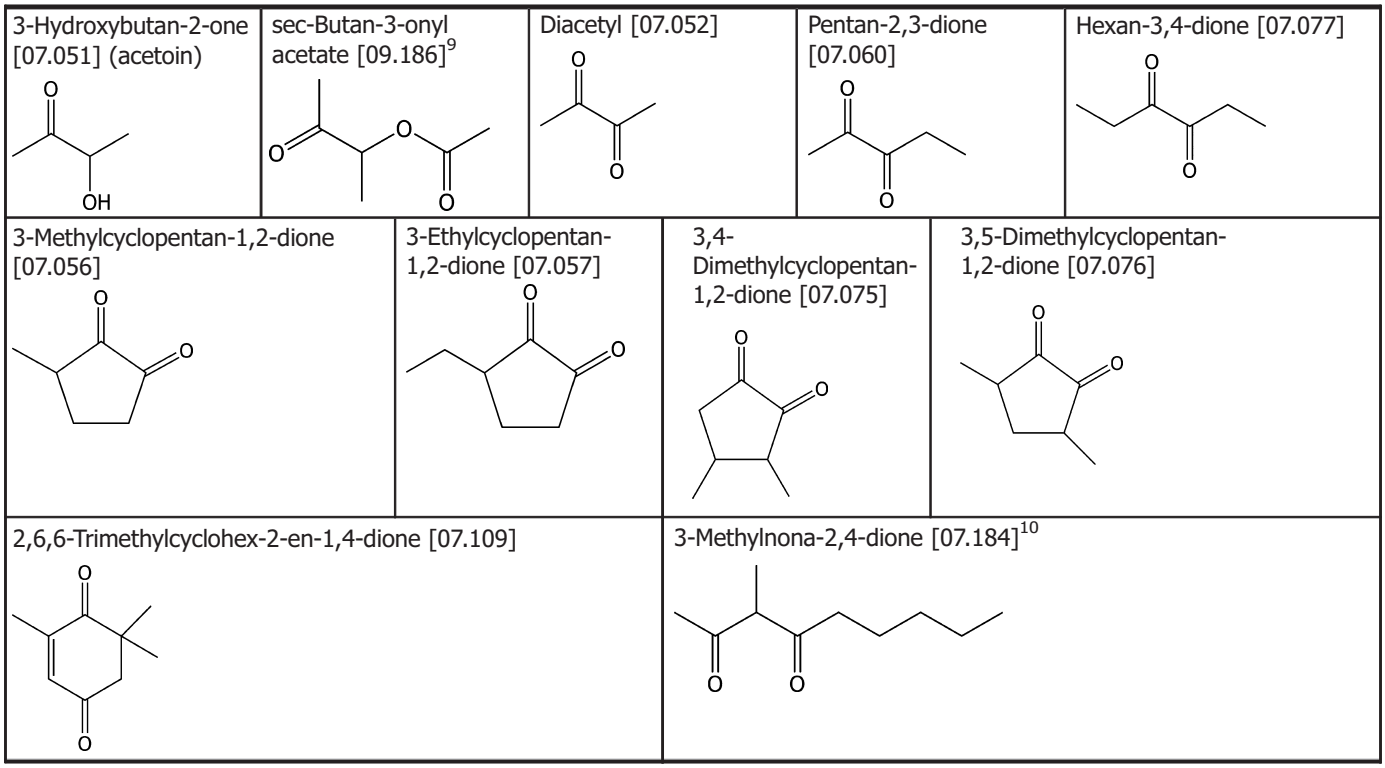

Figure 1: Molecular structures and [FLAVIS numbers] of the 11 flavouring compounds under assessment

The stereochemical configuration is not taken into account in the structures shown in Figure 1 due to various isomers resulting from the keto-enolic tautomerism of the majority of the compounds.

Table 1: Chemical Abstracts Service (CAS) and FLAVIS numbers and some characteristics of the 11 flavouring compounds under assessment

EU register name	CAS No	FLAVIS no	Molecular formula	Molecular weight	Physical state	Log K_{ow} (1)
3-Hydroxybutan-2-one	513-86-0	07.051	$C_4H_8O_2$	88.11	Liquid	−0.36
Diacetyl	431-03-8	07.052	$C_4H_6O_2$	86.09	Liquid	−1.34
3-Methylcyclopentan-1,2-dione	80-71-7	07.056	$C_6H_8O_2$	112.13	Solid	0.30
3-Ethylcyclopentan-1,2-dione	21835-01-8	07.057	$C_7H_{10}O_2$	126.16	Solid	0.83
Pentan-2,3-dione	600-14-6	07.060	$C_5H_8O_2$	100.12	Liquid	−0.85
3,4-Dimethylcyclopentan-1,2-dione	13494-06-9	07.075	$C_7H_{10}O_2$	126.12	Solid	0.53
3,5-Dimethylcyclopentan-1,2-dione	13494-07-0	07.076	$C_7H_{10}O_2$	126.12	Solid	0.53
Hexan-3,4-dione	4437-51-8	07.077	$C_6H_{10}O_2$	114.14	Liquid	−0.35
2,6,6-Trimethylcyclohex-2-en-1,4-dione	1125-21-9	07.109	$C_9H_{12}O_2$	152.2	Solid	0.76
3-Methylnona-2,4-dione	113486-29-6	07.184	$C_{10}H_{18}O_2$	170.25	Liquid	2.81
sec-Butan-3-onyl acetate	4906-24-5	09.186	$C_6H_{12}O_3$	130.14	Liquid	0.68

EU: European Union; FLAVIS number: EU Flavour Information System numbers.
(1): Logarithm of octanol–water partition coefficient.

[9] (R)- or (S)- isomer not specified.
[10] Racemate.

These substances are produced by chemical synthesis. Routes of synthesis are described in the dossier only for five of the 11 compounds under assessment. However, all six of the compounds for which routes of synthesis were not made available, show a high degree of purity.[11]

Batch-to-batch variation data were provided for five batches of each additive except 3,5-dimethylcyclopentan-1,2-dione [07.076], 3-methylnona-2,4-dione [07.184] and sec-butan-3-onyl acetate [09.186] for which only one, three and four batches, respectively, were provided owing to the low use volume.[12] The content of the active substance for all compounds exceeded the JECFA specifications (Table 2), except for 3-ethylcyclopentan-1,2-dione [07.057]. Data for this compound is provided only for a formulated product which contains 40% propylene glycol by weight. Propylene glycol is a recognised food additive (E 1520) and is used as a solvent/carrier for flavourings.[13] Both JECFA (WHO, 1974) and the Scientific Committee on Food (SCF; European Commission, 1978, 1986) assessed propylene glycol. An ADI of 25 mg/kg body weight (bw) per day was established by JECFA and considered acceptable by the SCF for the use of propylene glycol in materials in contact with food (EC, 1986).

Table 2: Identity of the substances and data on purity

EU register name	FLAVIS no	JECFA specification minimum %(1)	Assay %	
			Average	Range
3-Hydroxybutan-2-one	07.051	> 96	98.6	97.0–100
Diacetyl	07.052	> 95	99.6	98.8–99.8
3-Methylcyclopentan-1,2-dione	07.056	> 95	99.9	99.7–100
3-Ethylcyclopentan-1,2-dione	07.057	> 90	62.0(2)	61.5–62.5
Pentan-2,3-dione	07.060	> 93	98.6	98.5–99.1
3,4-Dimethylcyclopentan-1,2-dione	07.075	> 98	99.8	99.7–100
3,5-Dimethylcyclopentan-1,2-dione	07.076	> 97	97.8(3)	97.8
Hexan-3,4-dione	07.077	> 97	97.7	97.2–98.3
2,6,6-Trimethylcyclohex-2-en-1,4-dione	07.109	> 98	99.0	99.0–99.3
3-Methylnona-2,4-dione	07.184	> 97	97.7(4)	97.2–98.3
sec-Butan-3-onyl acetate	09.186	> 98	99.8(5)	99.2–100

FLAVIS number: EU Flavour Information System numbers; JECFA: The Joint FAO/WHO Expert Committee on Food Additives.
(1): FAO, 2006.
(2): The product is diluted in propylene glycol 40%.
(3): One batch, use of the product 1 kg/year or less.
(4): Three batches only, use of the product 1 kg/a or less.
(5): Four batches only, use of the product 1 kg/a or less.

Potential contaminants are considered as part of the product specification and are monitored as part of the Hazard Analysis and Critical Control Point procedure applied by all consortium members. The parameters considered include residual solvents, heavy metals and other undesirable substances. However, no evidence of compliance was provided for these parameters.

3.1.2. Stability

The shelf-life for the compounds under assessment ranges from 6 to 24 months when stored in closed containers under recommended conditions. This assessment is made on the basis of compliance with the original specification over this storage period.

3.1.3. Conditions of use

The applicant proposes the use of all of the 11 additives in feed for all animal species without withdrawal. For diacetyl [07.052], the applicant proposes a normal use level of 5 mg/kg feed and a high use level of 25 mg/kg. For the remaining 10 additives, the applicant proposes a normal use level of 1 mg/kg feed and a high use level of 5 mg/kg.

[11] Technical dossier/Section II.
[12] Technical dossier/Section II/Annex 2.1 and Supplementary information May 2011.
[13] Commission Regulation (EU) No 1130/2011 of the European Parliament and of the Council of 11 November 2011 amending Annex III to Regulation (EC) No 1338/2008 of the European Parliament and of the Council on food additives establishing a Union list of food additives approved for use in food additives, food enzymes, food flavourings and nutrients. OJ L 295, 12.11.2011, p. 178.

3.2. Safety

The assessment of safety is based on the highest use level proposed by the applicant (25 mg/kg complete feed for diacetyl and 5 mg/kg complete feed for the remaining compounds).

3.2.1. Absorption, distribution, metabolism and excretion (ADME)

Compounds belonging to CG 10 are absorbed from the gastrointestinal tract (Gabriel et al., 1972) and share common pathways of metabolism: (i) hydrolysis of esters by carboxylesterases, (ii) reduction of ketones to alcohols, (iii) oxidation of alcohols to acids, (iv) α–hydroxylation of the terminal methyl group to yield corresponding ketocarboxylic acids, (v) oxidative decarboxylation to yield carbon dioxide and an aliphatic carboxylic acid, and (vi) conjugation of α-hydroxyketones or their diol metabolites with glucuronic acid (WHO, 1999, 2000; EFSA CEF Panel, 2014c).

Aliphatic acyclic diketones [07.052, 07.060 and 07.077] and α–hydroxyketones [07.051], which contain a carbonyl function at the 2-position (i.e. a methyl ketone) are expected to undergo α–hydroxylation and subsequent oxidation of the terminal methyl group to eventually yield corresponding ketocarboxylic acids. These compounds are intermediary metabolites (e.g. α-ketoacids), which may undergo oxidative decarboxylation to yield carbon dioxide and an aliphatic carboxylic acid. The acid is then metabolised via β-oxidation and the citric acid cycle. β-Ketoacids and derivatives readily undergo decarboxylation to yield breakdown products, which are incorporated into normal biochemical pathways (EFSA, 2008a). Alternatively, the methyl-substituted diketones may be successively reduced to the corresponding hydroxyketones and diols, which are excreted in the urine as glucuronic acid conjugates. This pathway is favoured at elevated *in vivo* concentrations, especially for longer chain length ketones. If the carbonyl function is located elsewhere on the chain, reduction is the predominant pathway. α-Hydroxyketones or their diol metabolites may be excreted as glucuronic acid conjugates (WHO, 1999).

Low concentrations of aliphatic acyclic methyl ketones are mainly metabolised by oxidation of the terminal methyl group. At higher concentrations, acyclic α-diketones are metabolised via a reduction pathway to the diol and subsequent conjugation with glucuronic acid (WHO, 1999; EFSA CEF Panel, 2014b; FGE.09Rev5).

In rats and mice, orally administered acetoin (3-hydroxybutan-2-one [07.051]) is rapidly absorbed from the gastrointestinal tract (Gabriel et al., 1972). Upon intraperitoneal injection of acetoin-2,3-^{14}C to albino rats, $^{14}CO_2$ (representing 15% of the original dose) appeared in the expired air. Acetoin is metabolised primarily via oxidation at low concentrations *in vivo* and by reduction to 2,3-butanediol (butane-2,3-diol) at high concentrations. It is estimated that the rat liver is capable of oxidising 86 μg (1 μmol) acetoin/g liver per day (Gabriel et al., 1972).

Otsuka et al., 1996, demonstrated the high activities of diacetyl- and acetoin-reducing enzymes, in homogenate tissues of rats, especially in the liver, but also in the kidney and brain. One hour after oral administration of diacetyl to rats the amount of the compound in the liver, kidney and brain was 0.03%. Diacetyl was reduced to acetoin, which was mainly present in the brain. 2,3-Butanediol was also present in the three organs, amounting to about 2.3% of the administered dose. When acetoin was orally administered, it was also interconverted into diacetyl and 2,3-butanediol, being mainly present in the brain.

Diacetyl and acetoin are reported to be formed endogenously in humans and cats when pyruvate is converted to diacetyl and acetoin by pyruvate decarboxylase (Gabriel et al., 1972).

The major metabolic pathway for cyclopentanones was demonstrated in rabbits to involve the reduction of the ketone to the corresponding secondary alcohol followed by conjugation of the alcohol with glucuronic acid (Belsito et al., 2012). After oral gavage of cyclopentanone (193 mg/kg body weight), approximately half of the administered dose was excreted in the urine as the glucuronide of cyclopentanol. Small amounts of sulfur-containing metabolites were also detected in the urine representing about 5% of the administered dose. These were reported as an unidentified sulfur-containing metabolite (probably the sulfate ester of hydroxycycloalkylmercapturic acid), an ethereal sulfate and traces of *cis*- and *trans*-2-hydroxycyclopentylmercapturic acid. The unidentified sulfur-containing metabolite and 2-hydroxycyclopentylmercapturic acids were also detected in a similar study with rats (dose not given), but no glucuronide was found. In rats, the addition of glutathione resulting in the formation of 2-hydroxycyclopentylmercapturic acid (Belsito et al., 2012) and other sulfur-containing metabolites appears the main route of excretion. On the other hand, Cronholm (1974) detected in urine and bile of rats the glucuronyl metabolites of about 100% of cyclohexanone 24 h after its administration by gavage.

Metabolism studies of compounds belonging to CG 10 in animals, other than rats and rabbits, are lacking in the scientific literature. Carboxylesterases, responsible for the hydrolysis of esters, are present in the gut especially of ruminants and the liver of several animal species (cattle, pigs, chickens, rabbits and horses), operating the hydrolysis of esters and originating the respective alcohols and acids (Gusson et al., 2006). Carboxylesterase activity also plays a significant role in detoxification processes in fish (Li and Fan, 1997; Di Giulio and Hinton, 2008). Reduction of ketones to alcohols can also be carried out by carbonyl reductases that are widely distributed in animal species, including cattle, pig, rabbit, dog, sheep and birds (Felsted and Bachur, 1980), and more recently evaluated *in vitro* in the liver from cattle, pig, goat and sheep (Szotakova et al., 2004). Oxidative metabolism of xenobiotics is common in all animal species. The CYP450 monooxygenase families are present and have been characterised in a number of food-producing animals, including ruminants, horses, pigs, (Nebbia et al., 2003; Ioannides, 2006; Fink-Gremmels, 2008), fish (Wolf and Wolfe, 2005) and birds (Blevins et al., 2012). All these species also carry out conjugation reactions with sulfate and glucuronic acid (Watkins and Klaassen, 1986; James, 1987; Gusson et al., 2006), producing water-soluble derivatives that are eliminated in urine. Therefore, mammals, fish and birds, can also be assumed to have the ability to metabolise and excrete the flavouring substances from CG 10 and there is no evidence that they or their metabolites would accumulate in tissues and cause a concern for consumer safety. The FEEDAP Panel notes that for feline species the capacity for conjugation is limited (Shrestha et al., 2011; Court, 2013).

3.2.2. Toxicological studies

Subchronic repeated-dose studies with multiple doses tested could be found for 3-hydroxybutan-2-one [07.051], diacetyl [07.052], 3,4-dimethylcyclopentan-1,2-dione [07.075] and 3,5-dimethylcyclopentan-1,2-dione [07.076]. For 3-ethylcyclopentan-1,2-dione [07.057], a chronic study was available. An additional study was identified in which hexan-3,4-dione [07.077] was tested as an admixture with 3-hexanone (15%) at a single dose level. Based on the chemical structure, 3-hydroxybutan-2-one, diacetyl and hexan-3,4-dione are acyclic compounds, the others compounds are cyclopentanediones.

In a 13-week study in rats (males/females, 15 animals/group), 3-hydroxybutan-2-one [07.051] was administered with the diet at doses of 0, 85, 330 and 1,345 mg/kg bw per day. No treatment-related effects on body weight gain, haematological and urinary parameters, serum chemistry, organ weight and histopathology were seen up to 330 mg/kg bw per day. Several effects were observed at the highest dose tested, i.e. a reduction in body weight gain associated with a reduction in food and water consumption, an increase in relative liver weight and a slight anaemia. From this study, a no observed adverse effect level (NOAEL) of 330 mg/kg bw per day could be derived (Gaunt et al., 1972).

A NOAEL of 90 mg/kg bw per day was derived from a 13-week study in rats (15 males/15 females each group), in which diacetyl [07.052] was administered by gavage at nominal doses of 0, 10, 30, 90 and 540 mg/kg bw per day. No adverse effects were seen at the three low doses tested on haematological and urinary parameters, serum chemistry, absolute and relative organ weight and histopathology. Several effects were observed at the highest dose tested (540 mg/kg bw), i.e. a decrease in weight gain associated with an increase in water consumption, anaemia, increased leucocyte count, increased relative weights of the liver, kidneys, adrenals and pituitary glands. At the same dose, stomach lesions seen at necropsy revealed necrosis with infiltration by inflammatory cells (Colley et al., 1969).

A repeated-dose toxicity study (90 days, only one dose tested) in rats was available for hexan-3,4-dione [07.077] containing about 15% 3-hexanone (Posternak et al., 1969). The study considered a number of endpoints (body weight, feed intake; haematology and clinical chemistry; gross pathology and histopathology) and showed no effects at the dose tested, i.e. 17.47 and 17.34 mg/kg bw per day in male and female rats, respectively. The NOAEL for this study is 17.34 mg/kg bw per day, the only dose tested.

The FEEDAP Panel retains a NOAEL of 90 mg/kg bw per day derived from the 90-day study with diacetyl [07.052] and applies it as a group NOAEL for 3-hydroxybutan-2-one [07.051] and its ester [09.186], pentan-2,3-dione [07.060] and hexan-3,4-dione [07.077] on the basis of structure similarity and common metabolism.

A trial was conducted to assess the chronic toxicity of 3-ethylcyclopentan-1,2-dione [07.057] on reproduction and development in rats (male and female Charles River CD-COBS) following administration to three successive generations (King et al., 1979, unpublished). In each generation, rats received diet containing 3-ethylcyclopentan-1,2-dione corresponding to dose levels of 0 (untreated controls), 0 (propylene glycol vehicle), 30, 80, and 200 mg/kg body weight/day. The F0 group (20 animals/sex/treatment) entered the study at weaning and were mated on day 64. Animals from the control groups and the high-dose group were maintained on trial for 12 months. The F1 generation

(50 animals/sex per treatment except control, 100 animals/sex) was exposed to the test substance *in utero*, via milk until weaning and then through the diet for a further 23 months. The final examination of the F1 generation included ophthalmology, clinical chemistry, haematology and a full histopathology. The F1 generation was bred twice (days 99 and 155) and 20 litters/treatment group from the first mating selected to provide the F2 generation which were in turn mated at day 84. The F3 generation were killed after weaning. Survival, food consumption, growth, reproductive performance, haematological and clinical chemistry parameters were not adversely affected. Gross pathological and histopathological examination revealed no significant treatment-related effects. The incidence of benign or malignant tumours in treated animals was not significantly different to that in controls in the F0 and F1 generations. From this study, it is concluded that ethylcyclopentan-1,2-dione [07.057] was not carcinogenic in rats under the study conditions and that a NOAEL of 200 mg/kg body weight (the highest dose tested) can be derived for chronic and developmental effects.

In a 13-week study in male rats (10 animals each group), 3,4-dimethylcyclopentan-1,2-dione [07.075] was administered via the diet at nominal doses of 0, 400, 4,000 and 12,900 mg/kg corresponding to 0, 20, 200 and 645 mg/kg bw per day. The study considered a number of endpoints (mortality, body weight, feed intake; haematology; gross pathology and histopathology). A depression of food intake and a decrease in body weight gain were seen in animals exposed to the highest dose group (> 10% reduction). Since the efficiency of feed conversion was unaffected the authors attributed this to the sensory properties of the diet leading to inappetence. No other changes were observed, which led the authors to retain the highest dose tested as the NOAEL for the study (Wheldon and Krajkeman, 1967, unpublished; RIFM database). In the same report, a NOAEL of 610 mg/kg bw per day was identified for 3,5-dimethylcyclopentan-1,2-dione [07.076]. However, this compound also induced reduced feed intake and growth at the highest dose tested (12,200/24,000 mg/kg diet). Since significant growth reduction is considered adverse the panel opts for the middle dose tested from which a NOAEL of 200 mg/kg body weight can be derived for 3,4-dimethylcyclopentan-1,2-dione and a NOAEL of 500 mg/kg body weight for 3,5-dimethylcyclopentan-1,2-dione.

Secondary references referred to a repeated dose toxicity study (90 days, one dose tested) in rat (15 males/15 females) with 3-methylcyclopentan-1,2-dione [07.056] in which a NOAEL of 500 mg/kg bw per day (corresponding to 1%) was derived (Dow chemical, unpublished, 1953 as described in RIFM report, 1976). However, the study report was not available and the NOAEL could not be confirmed.

The FEEDAP Panel retains the more conservative NOAEL of 200 mg/kg bw per day derived from the combined developmental/carcinogenicity study with 3-ethylcyclopentan-1,2-dione [07.057] and applies it as a group NOAEL for cyclopentanediones.

3.2.3. Safety for the target species

The first approach to the safety assessment for target species takes account of the applied use levels in animal feed relative to the maximum reported exposure of humans on the basis of the metabolic body weight. The data for human exposure in the EU (EFSA, 2008a, 2009, EFSA CEF Panel, 2014b,c) ranges from 0.024 to 2,300 μg/person per day, corresponding to 0.011–106.7 μg/kg$^{0.75}$ per day. Table 3 summarises the result of the comparison with human exposure for representative target animals. The body weight of target animals is taken from the default values shown in Table 4.

Table 3: Comparison of exposure of humans and target animals to the flavourings under application

EU register name	Use level in feed (mg/kg)	Human exposure (μg/kg bw$^{0.75}$ per day)(1)	Target animal exposure (μg/kg bw$^{0.75}$ per day)		
			Salmon	Piglet	Dairy cow
3-Hydroxybutan-2-one	5	107	118	526	777
Diacetyl	25	102	588	2,632	3,885
3-Methylcyclopentan-1,2-dione	5	26.4	118	526	777
3-Ethylcyclopentan-1,2-dione	5	1.48	118	526	777
Pentan-2,3-dione	5	6.03	118	526	777
3,4-Dimethylcyclopentan-1,2-dione	5	1.39	118	526	777
3,5-Dimethylcyclopentan-1,2-dione	5	1.62	118	526	777
Hexan-3,4-dione	5	0.97	118	526	777
2,6,6-Trimethylcyclohex-2-en-1,4-dione	5	2.32	118	526	777

EU register name	Use level in feed (mg/kg)	Human exposure (μg/kg bw$^{0.75}$ per day)(1)	Target animal exposure (μg/kg bw$^{0.75}$ per day)		
			Salmon	Piglet	Dairy cow
3-Methylnona-2,4-dione	5	0.016	118	526	777
sec-Butan-3-onyl acetate	5	0.0011	118	526	777

bw: body weight.
(1): Metabolic body weight (kg bw$^{0.75}$) for a 60-kg person = 21.6.

Table 3 shows that for all compounds the intake by the target animals exceeds that of humans resulting from use in food. As a consequence, safety for the target species at the feed concentration applied cannot be derived from the risk assessment for food use.

As an alternative, the maximum feed concentration considered as safe for the target animal can be derived from the lowest NOAEL available. Toxicological data, from which a NOAEL value could be derived, were available for three acyclic compounds (3-hydroxybutan-2-one [07.051], diacetyl [07.052] and hexan-3,4-dione [07.077]) and four cyclopentadiones (3-methylcyclo-pentan-1,2-dione [07.056], 3-ethylcyclopentan-1,2-dione [07.057], 3,4-dimethylcyclopentan-1,2-dione [07.075] and 3,5-dimethyl cyclopentan-1,2-dione [07.076]) (see Section 3.2.2). For acyclic compounds, a group NOAEL of 90 mg/kg bw per day derived from the 90-day study with diacetyl [07.052] was considered to apply also to 3-hydroxybutan-2-one [07.051] and its ester [09.186], pentan-2,3-dione [07.060] and hexan-3,4-dione [07.077]. For cyclopentanediones, the more conservative NOAEL of 200 mg/kg bw per day derived from the combined developmental/carcinogenicity study with 3-ethylcyclopentan-1,2-dione [07.057] was applied as a group NOAEL.

Applying an uncertainty factor (UF) of 100 to these NOAELs, the maximum safe intake for the target species was derived for the eight compounds following the EFSA Guidance for sensory additives (EFSA FEEDAP Panel, 2012a), and thus the maximum safe feed concentration was calculated. The results are summarised in Table 4. The UF for cats is increased by an additional factor of 5 because of the reduced capacity of glucuronidation (Court and Greenblatt, 1997).

Table 4: Maximum safe concentration in feed for different target animals for (**A**) acyclic compounds (NOAEL 90 mg/kg bw per day) and (**B**) cyclopentadiones (NOAEL 200 mg/kg bw per day)

Target animal	Default values		Maximum safe intake/feed concentration			
	Body weight (kg)	Feed intake (g/day)(1)	Intake (mg/day)		Concentration (mg/kg feed)(2)	
			A	B	A	B
Salmonids	2	40	1.8	4	45	101
Veal calves (milk replacer)	100	2,000	90	200	45	100
Cattle for fattening	400	8,000	360	800	40	88
Dairy cows	650	20,000	585	1,300	26	57
Piglets	20	1,000	18	40	18	40
Pigs for fattening	100	3,000	90	200	30	67
Sows	200	6,000	180	400	30	67
Chickens for fattening	2	120	1.8	4	15	33
Laying hens	2	120	1.8	4	15	33
Turkeys for fattening	12	400	10.8	24	27	60
Dogs	15	250	13.5	30	48	106
Cats(3)	3	60	0.5	1.2	8	18

NOAEL: no observed adverse effect level; bw: body weight.
(1): Complete feed with 88% dry matter (DM), except milk replacer for veal calves (94.5% DM), and for cattle for fattening, dairy cows, dogs and cats for which the values are DM intake.
(2): Complete feed containing 88% DM, milk replacer 94.5% DM.
(3): The uncertainty factor for cats is increased by an additional factor of 5 because of the reduced capacity of glucuronidation.
(**A**): 3-Hydroxybutan-2-one [07.051], diacetyl [07.052], hexan-3,4-dione [07.077], pentan-2,3-dione [07.060] and sec-butan-3-onyl acetate [09.186].
(**B**): 3-Methylcyclopentan-1,2-dione [07.056], 3-ethylcyclopentan-1,2-dione [07.057] 3,4-dimethylcyclopentan-1,2-dione [07.075] and 3,5-dimethylcyclopentan-1,2-dione [07.076].

For the two remaining compounds, 2,6,6-trimethylcyclohex-2-en-1,4-dione [07.109] and 3-methylnona-2,4-dione [07.184], adequate subchronic, repeated-dose studies performed with the additive under assessment were not available. Therefore, the threshold of toxicological concern (TTC) approach was followed to derive the maximum safe feed concentration (EFSA FEEDAP Panel, 2012a).

For these two compounds belonging to Cramer Class II compounds, the calculated safe use level for these compounds is 0.5 mg/kg complete feed for cattle, salmonids and non-food producing animals and 0.3 mg/kg complete feed for pigs and poultry.

3.2.3.1. Conclusions on safety for the target species

The FEEDAP Panel concludes that for:

- diacetyl [07.052] is safe at the proposed maximum use level of 25 mg/kg complete feed for all target species, except piglets, chickens for fattening, laying hens and cats, for which the proposed normal use level of 5 mg/kg is safe;
- 3-hydroxybutan-2-one [07.051], 3-methylcyclo-pentan-1,2-dione [07.056], 3-ethylcyclopentan-1,2-dione [07.057], pentan-2,3-dione [07.060], 3,4-dimethylcyclopentan-1,2-dione [07.075], 3,5-dimethyl cyclopentan-1,2-dione [07.076], hexan-3,4-dione [07.077] and sec-butan-3-onyl acetate [09.186] are safe at the proposed maximum dose level of 5 mg/kg complete feed for all target species;
- 2,6,6-trimethylcyclohex-2-en-1,4-dione [07.109] and 3-methylnona-2,4-dione [07.184] are safe only at concentrations of 0.5 mg/kg complete feed for cattle, salmonids and non-food producing animals and 0.3 mg/kg complete feed for pigs and poultry.

3.2.4. Safety for the consumer

The safety for the consumer of the compounds in CG 10, used as food flavours, has already been assessed by JECFA (WHO, 1999, 2000) and EFSA (EFSA 2008a, 2009; EFSA CEF Panel, 2011, 2014a,b, c). All these compounds are presently authorised as food flavourings without limitations.[5]

Given the use levels of CG 10 compounds to be applied in feed, their hydrophilic properties and the expected extensive metabolism and excretion in target animals (see Section 3.2.1), the FEEDAP Panel considers that the possible residues in food derived from animals fed with these flavourings would not appreciably increase the human intake of these compounds. Consequently, no safety concern would arise for the consumer from the use of these 11 compounds up to the highest safe level in feeds.

3.2.5. Safety for the user

No specific data on the safety for the user were provided. In the material safety data sheets[14] hazards for skin and eye contact and respiratory exposure are recognised for the majority of the compounds under application. Most are classified as irritating to the respiratory system. In particular, respiratory exposure to diacetyl has been demonstrated to be harmful for exposure at the workplace (review by NIOSH, 2011; Shibamoto, 2014).

3.2.6. Safety for the environment

The additions of naturally occurring substances that will not result in a substantial increase in the concentration in the environment are exempt from further assessment. Examination of the published literature shows that this applies to four substances, namely, 3-hydroxybutan-2-one [07.051], diacetyl [07.052], hexan-3,4-dione [07.077] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [07.109], which occur in the environment at levels above the application rate of 25 (for diacetyl) and 5 mg/kg feed for the remaining three compounds (data taken from the Netherlands Organisation for Applied Scientific Research (TNO) database Volatile Compounds in Food *ver.* 14.1; Burdock, 2003).[15]

The other seven compounds, namely 3-methylcyclopentan-1,2-dione [07.056], 3-ethylcyclopentan-1,2-dione [07.057], pentan-2,3-dione [07.060], 3,4-dimethylcyclopentan-1,2-dione [07.075], 3,5-dimethylcyclopentan-1,2-dione [07.076], 3-methylnona-2,4-dione [07.184] and sec-butan-3-onyl acetate [09.186], could not be shown to occur in the environment at levels above the application rate of 5 mg/kg feed for the remaining three compounds. However, the FEEDAP Panel assumes that there is a high

[14] Technical dossier/Section II/Annex II.3.
[15] Technical dossier/Supplementary information June 2011.

probability of complete hydrolysis in the target animal of the ester sec-butan-3-onyl acetate [09.186], resulting in acetic acid and 3-hydroxybutan-2-one [07.051], which are naturally occurring compounds. Similarly, considering the metabolism in the target animals (see Section 3.2.1), the FEEDAP Panel assumes that pentan-2,3-dione [07.060] and 3-methylnona-2,4-dione [07.184] will be completely metabolised in the target animals. Therefore, these compounds are excluded from further assessment.

For the remaining four compounds, namely 3-methylcyclopentan-1,2-dione [07.056], 3-ethylcyclopentan-1,2-dione [07.057], 3,4-dimethylcyclopentan-1,2-dione [07.075] and 3,5-dimethylcyclopentan-1,2-dione [07.076], the predicted environmental concentration for soil (PEC_{soil}) was calculated based on the use rate (Table 5) and compared with the trigger values for compartments set in the phase I of the relevant EFSA guidance (EFSA, 2008b).

Table 5: Predicted environmental concentration (PEC) values of the four flavourings of CG 10 under assessment (calculated for lamb manure)

EU register name	CAS no.	Dose mg/kg	PEC_{soil} (µg/kg)	$PEC_{porewater}$ (µg/L)	$PEC_{surfacewater}$ (µg/L)
3-Methylcyclopentan-1,2-dione	80-71-7	5	107	765	255
3-Ethylcyclopentan-1,2-dione	21835-01-8	5	107	671	224
3,4-Dimethylcyclopentan-1,2-dione	13494-06-9	5	107	788	263
3,5-Dimethylcyclopentan-1,2-dione	13494-07-0	5	107	788	263

EU: European Union; CAS no: Chemical Abstracts Service.

PEC_{soil} values are above the threshold of 10 µg/kg (EFSA, 2008b). The PEC for pore water is dependent on the sorption, which is different for each compound. For these calculations, the substance-dependent constants organic carbon sorption constant (K_{oc}), molecular weight, vapour pressure and solubility are needed. These were estimated from the Simplified Molecular Input Line Entry Specification (SMILES) notation of the chemical structure using EPIWEB 4.1 (Table 6).[16] This program was also used to derive the SMILES notation from the CAS numbers. The K_{oc} value derived from the first-order molecular connectivity index was used, as recommended by the EPIWEB program.

Table 6: Physicochemical properties predicted by EPIWEB 4.1 for the four flavourings of CG 10 under assessment

EU register name	CAS no.	Predicted by EPIWEB 4.1				
		DT_{50}(1) (days)	Molecular weight (g/mol)	Vapour pressure (Pa)	Solubility (mg/L)	K_{oc}(2) (L/kg)
3-Methylcyclopentan-1,2-dione	80-71-7	7	112.13	0.1	8,501	1.2
3-Ethylcyclopentan-1,2-dione	21835-01-8	7	126.16	0.1	2,878	2.3
3,4-Dimethylcyclopentan-1,2-dione	13494-06-9	10	126.16	25	121,100	1.0
3,5-Dimethylcyclopentan-1,2-dione	13494-07-0	10	126.16	25	121,100	1.0

EU: European Union; CAS no: Chemical Abstracts Service.
(1): DT_{50}: half-life of the additive (EPIWB 4.1.BioWin4.1).
(2): K_{oc}: organic carbon sorption constant (EPIWB 4.1.KocWin2.0).

The half-life (DT_{50}) was calculated using BioWin4.1 (Ultimate Survey Model), which gives a rating number. This rating number *r* was translated into a half-life using the formula by Arnot et al. (2005):

$$DT_{50} = 10^{(-r \times 1.07 + 4.12)}$$

This is the general regression used to derive estimates of aerobic environmental biodegradation half-lives from BioWin 4.1 model output.

The calculated predicted concentrations for groundwater ($PEC_{porewater}$) for all four substances are above 0.1 µg/L and for soil (PEC_{soil}) above 10 µg/kg (see Table 5). Therefore, they are subjected to phase II risk assessment.

In the absence of experimental data, the phase II risk assessment was performed using ECOSAR v1.11, which estimates the half-maximal effective concentration (EC_{50}) or lethal concentration (LC_{50}) for ecotoxicologically relevant organisms from the SMILES notation of the substance. The predicted PNEC for

[16] Available online: http://www.epa.gov/opptintr/exposure/pubs/episuitedl.htm

aquatic compartment ($PNEC_{aquatic}$) was derived from the lowest toxicity value for freshwater environment by applying a UF of 1,000.

Table 7: Phase II environmental risk assessment of aquatic compartment for CG 10 compounds used as feed additives for terrestrial farm animals (exposure and effect data were modelled using EPIWEB 4.1 and ECOSAR 1.11)

EU Register name Aquatic	LC_{50} (1) Fish (mg/L)	LC_{50} (1) Daphnids (mg/L)	EC_{50} (2) Algae (mg/L)	$PNEC_{aquatic}$ (µg/L)	PEC_{sw} (3) (µg/L)	PEC_{sw}/ $PNEC_{sw}$
3-Methylcyclopentan-1,2-dione	398	331	169	169	255	1.5
3-Ethylcyclopentan-1,2-dione	189	135	76.0	76.1	224	2.9
3,4-Dimethylcyclopentan-1,2-dione	8351	3898	1291	76.1*	263	3.5
3,5-Dimethylcyclopentan-1,2-dione	8351	3898	1291	76.1*	263	3.5

EU: European Union; PNEC: predicted no effect concentration.
(1): LC_{50}: the concentration of a test substance which results in a 50% mortality of the test species.
(2): EC_{50}: the concentration of a test substance which results in 50% of the test animals being adversely affected (i.e. both mortality and sublethal effects).
(3): PEC_{sw}: predicted environmental concentration in surface water.
*: The LC_{50} for algae of 3-ethylcyclopentan-1,2,dione was taken to derive a PNEC.

For 3,4-dimethylcyclopentan-1,2-dione [07.075] and 3,5-dimethylcyclopentan-1,2-dione [07.076], no proper quantitative structure–activity relationship (QSAR) were available and therefore QSAR for the class 'neutral organic' was used by ECOSAR. This default model is not the most appropriate since these compounds are very similar to the other two cyclopentanediones, 3-methylcyclopentan-1,2-dione [07.056] and 3-ethylcyclopentan-1,2-dione [07.057], for which the vinyl/allylketones QSAR was used. The QSAR for vinyl/alkylketones is considered more relevant for all cyclopentanediones. Therefore, the $PNEC_{aquatic}$ for 3-ethylcyclopentan-1,2,dione [07.057] was taken in preference as a worst-case estimate.

Concerning the fresh water environment, the maximum proposed use level (5 mg/kg) would result in PEC_{sw}/PNEC ratio > 1 for all compounds (Table 7), whereas the proposed normal use level of 1 mg/kg would not cause a risk for this compartment (PEC_{sw}/PNEC ratio in the range 0.301–0.691).

It was not possible to obtain toxicity data for earthworms using ECOSAR for any of the compounds in Table 7. Therefore, the equilibrium partitioning method was applied, which assumes that earthworms do not show higher sensitivity than aquatic organisms. To determine the potential exposure of earthworms, the pore water concentration is set as three times higher than the surface water concentration (EFSA, 2008b). The resulting PEC/PNEC ratio is equal or lower than 1 only when the use level is lower than 0.5 mg/kg.

If used in fish feed at the highest proposed use level of 5 mg/kg complete feed in land-based aquaculture systems, none of the additives under assessment would result in a predicted environmental concentration of the additive (parent compound) in surface water (PEC_{swaq}) above the trigger value of 0.1 µg/L as calculated according to the guidance (EFSA, 2008b). For sea cages, a dietary concentration of 0.047 mg/kg would ensure that the threshold for the predicted environmental concentration of the additive (parent compound) in sediment (PEC_{sed}) of 10 µg/kg is not exceeded when calculated according to the EFSA guidance (EFSA, 2008b).

3.2.6.1. Conclusions on safety for the environment

For 3-hydroxybutan-2-one [07.051], diacetyl [07.052], pentan-2,3-dione [07.060], hexan-3,4-dione [07.077], 2,6,6-trimethylcyclohex-2-en-1,4-dione [07.109], 3-methylnona-2,4-dione [07.184] and sec-butan-3-onyl acetate [09.186], the maximum proposed use levels are considered safe for the environment. For cyclopentanediones (3-methylcyclopentan-1,2-dione [07.056], 3-ethylcyclopentan-1.2-dione [07.057], 3,4 dimethylcyclopentan-1,2-dione [07.075] and 3,5-dimethylcyclopentan-1,2-dione [07.076]) usage at levels up to 0.5 mg/kg feed is unlikely to have an adverse effect on the terrestrial or freshwater environments.

3.3. Efficacy

Since all 11 compounds are used in food as flavourings and their function in feed is essentially the same as that in food no further demonstration of efficacy is necessary.

4. Conclusions

The FEEDAP Panel concludes that diacetyl [07.052] is safe at the proposed maximum use level of 25 mg/kg complete feed for all target species, except piglets, chickens for fattening, laying hens and cats, for which the proposed normal use level of 5 mg/kg is safe; 3-hydroxybutan-2-one [07.051], 3-methylcyclo-pentan-1,2-dione [07.056], 3-ethylcyclopentan-1,2-dione [07.057], pentan-2,3-dione [07.060], 3,4-dimethylcyclopentan-1,2-dione [07.075], 3,5-dimethyl cyclopentan-1,2-dione [07.076], hexan-3,4-dione [07.077] and sec-butan-3-onyl acetate [09.186] are safe at the proposed maximum dose level of 5 mg/kg complete feed for all target species; 2,6,6-trimethylcyclohex-2-en-1,4-dione [07.109] and 3-methylnona-2,4-dione [07.184], are safe only at concentrations of 0.5 mg/kg complete feed for cattle, salmonids and non-food producing animals and 0.3 mg/kg complete feed for pigs and poultry.

No safety concern would arise for the consumer from the use of these compounds up to the highest proposed level in feeds.

Hazards for skin and eye contact and respiratory exposure are recognised for the majority of the compounds under application. Most are classified as irritating to the respiratory system.

For 3-hydroxybutan-2-one [07.051], diacetyl [07.052], pentan-2,3-dione [07.060], hexan-3,4-dione [07.077], 2,6,6-trimethylcyclohex-2-en-1,4-dione [07.109], 3-methylnona-2,4-dione [07.184] and sec-butan-3-onyl acetate [09.186], the maximum proposed use levels are considered safe for the environment. For cyclopentanediones (3-methylcyclopentan-1,2-dione [07.056], 3-ethylcyclopentan-1.2-dione [07.057], 3,4 dimethylcyclopentan-1,2-dione [07.075] and 3,5-dimethylcyclopentan-1,2-dione [07.076]) usage at levels up to 0.5 mg/kg feed is unlikely to have an adverse effect on the terrestrial or freshwater environments.

Because all the compounds under assessment are used in food as flavourings and their function in feed is essentially the same as that in food, no further demonstration of efficacy is necessary.

Documentation provided to EFSA

1. Chemically defined flavourings from Flavouring Group 10 – Secondary aliphatic saturated or unsaturated alcohols/ketones/ketals/esters with a second secondary or tertiary oxygenated functional group for all animal species and categories. August 2010. Submitted by Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG).
2. Chemically defined flavourings from Flavouring Group 10 – Secondary aliphatic saturated or unsaturated alcohols/ketones/ketals/esters with a second secondary or tertiary oxygenated functional group for all animal species and categories. Supplementary information. May 2011. Submitted by Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG).
3. Chemically defined flavourings from Flavouring Group 10 – Secondary aliphatic saturated or unsaturated alcohols/ketones/ketals/esters with a second secondary or tertiary oxygenated functional group for all animal species and categories. Supplementary information. April 2012. Submitted by Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG).
4. Chemically defined flavourings from Flavouring Group 10 – Secondary aliphatic saturated or unsaturated alcohols/ketones/ketals/esters with a second secondary or tertiary oxygenated functional group for all animal species and categories. Supplementary information. July 2012. Submitted by Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG).
5. Chemically defined flavourings from Flavouring Group 10 – Secondary aliphatic saturated or unsaturated alcohols/ketones/ketals/esters with a second secondary or tertiary oxygenated functional group for all animal species and categories. Supplementary information. July 2016. Submitted by Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG).
6. Evaluation report of the European Union Reference Laboratory for Feed Additives on the methods(s) of analysis for Chemically Defined Flavourings – Group 10 (CDG 10 – Secondary aliphatic saturated or unsaturated alcohols/ketones/ketals/esters with a second secondary or tertiary oxygenated functional group).
7. Comments from Member States.

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Abbreviations

ADI	acceptable daily intake
bw	body weight
CAS	Chemical Abstracts Service
CD	Commission Decision
CEF	EFSA Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CG	chemical group
CDG	chemically defined group
DM	dry matter
DT_{50}	degradation half-time
EC_{50}	half-maximal effective concentration
ECOSAR	component program of EPI suite™
EEIG	European Economic Interest Grouping
EPI suite	Estimation Programs Interface (EPI) Suite™
EURL	European Union Reference Laboratory
FAO	Food and Agriculture Organization
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
FFAC	Feed Flavourings authorisation Consortium of (FEFANA) the EU Association of Specialty Feed Ingredients and their Mixtures
FGE	Flavouring Group Evaluation
FLAVIS	the EU Flavour Information System
FL-No	FLAVIS number
GC–MS	gas chromatography–mass spectrometry
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
K_{oc}	organic carbon sorption constant
K_{ow}	octanol–water partition coefficient
LC_{50}	lethal concentration 50
Log K_{ow}	logarithm of octanol–water partition coefficient
NOAEL	no observed adverse effect level
PEC	predicted environmental concentration
PEC_{swaq}	predicted environmental concentration of the additive (parent compound) in surface water
PNEC	predicted no effect concentration
QSAR	quantitative structure–activity relationship
SCF	Scientific Committee on Food
SMILES	Simplified Molecular Input Line Entry Specification
TNO	Netherlands Organisation for Applied Scientific Research
TTC	threshold of toxicological concern
UF	uncertainty factor
WHO	World Health Organization

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for secondary aliphatic saturated or unsaturated alcohols/ ketones/ketals/esters with a second secondary or tertiary oxygenated functional group

The *Chemically Defined Flavourings - Group 10* (*CDG10 - Secondary aliphatic saturated or unsaturated alcohols/ketones/ketals/esters with a second secondary or tertiary oxygenated functional group*), in this application comprises 11 substances, for which authorisation as feed additives is sought under the category "sensory additives", functional group 2(b) "flavouring compounds", according to the classification system of Annex I of Regulation (EC) No 1831/2003.

In the current application submitted according to Article 4(1) and Article 10(2) of Regulation (EC) No 1831/2003, the authorisation for all species and categories is requested. The flavouring compounds of interest have a purity ranging from 95% to 98% (90% for the *3-ethylcyclopentan-1,2-dione*).

Mixtures of flavouring compounds are intended to be incorporated only into *feedingstuffs* or drinking *water*. The Applicant suggested no minimum or maximum levels for the different flavouring compounds in *feedingstuffs*.

For the identification of volatile chemically defined flavouring compounds *CDG10* in the *feed additive,* the Applicant submitted a qualitative multi-analyte gas-chromatography mass-spectrometry (GC-MS) method, using Retention Time Locking (RTL), which allows a close match of retention times on GC-MS. By making an adjustment to the inlet pressure, the retention times can be closely matched to those of a reference chromatogram. It is then possible to screen samples for the presence of target compounds using a mass spectral database of RTL spectra. The Applicant maintained two FLAVOR2 databases/libraries (for retention times and for MS spectra) containing data for more than 409 flavouring compounds. These libraries were provided to the EURL. The Applicant provided the typical chromatogram for the *CDG10* of interest.

In order to demonstrate the transferability of the proposed analytical method (relevant for the method verification), the Applicant prepared a model mixture of flavouring compounds on a solid carrier to be identified by two independent expert laboratories. This mixture contained twenty chemically defined flavourings belonging to twenty different chemical groups to represent the whole spectrum of compounds in use as feed flavourings with respect to their volatility and polarity. Both laboratories properly identified all the flavouring compounds in all the formulations. Since the substances of *CDG10* are within the volatility and polarity range of the model mixture tested, the Applicant concluded that the proposed analytical method is suitable to determine qualitatively the presence of the substances from *CDG10* in the *mixture of flavouring compounds.*

Based on the satisfactory experimental evidence provided, the EURL recommends for official control for the qualitative identification in the *feed additive* of the individual (or mixture of) *flavouring compounds* of interest listed in Table 1 (*) the GC-MS-RTL (Agilent specific) method submitted by the Applicant.

As no experimental data were provided by the Applicant for the identification of the *active substance(s)* in *feedingstuffs* and *water*, no methods could be evaluated. Therefore the EURL is unable to recommend a method for the official control to identify the *active substance(s)* of interest listed in Table 1 (*) in *feedingstuffs* or *water*.

Further testing or validation of the methods to be performed through the consortium of National references Laboratories as specified by article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

(*)Full list provided in EURL evaluation report, available from the EURL website.

European Food Safety Authority

The EFSA Journal (2009) ON-879, 1-27

SCIENTIFIC OPINION

Flavouring Group Evaluation 213: alpha,beta-Unsaturated alicyclic ketones and precursors from chemical subgroup 2.7 of FGE.19[1]

Scientific Opinion of the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)

(Question No EFSA-Q-2008-768)

Adopted on 27 november 2008

PANEL MEMBERS

Arturo Anadon, David Bell, Mona-Lise Binderup, Wilfried Bursch, Laurence Castle, Riccardo Crebelli, Karl-Heinz Engel, Roland Franz, Nathalie Gontard, Thomas Haertle, Trine Husøy, Klaus-Dieter Jany, Catherine Leclercq, Jean Claude Lhuguenot, Wim Mennes, Maria Rosaria Milana, Karla Pfaff, Kettil Svensson, Fidel Toldra, Rosemary Waring, Detlef Wölfle.

SUMMARY

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) was asked to provide scientific advise for the Commission on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was asked to evaluate flavouring substances using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000.

The present Flavouring Group Evaluation 213 (FGE.213) concerns 26 substances. The 26 substances correspond to subgroup 2.7 of FGE.19. Twenty-three of the substances are alpha,beta-unsaturated alicyclic ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 16.044] and three are precursors for such ketones [FL-no: 02.106, 09.305 and 09.525].

[1] For citation purposes: Scientific Opinion of the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids on a request from the Comission on Flavouring Group Evaluation 213: alpha,beta-Unsaturated alicyclic ketones and precursors from chemical subgroup 2.7 of FGE.19. The EFSA Journal (2009) ON-879, 1-27.

The Panel concluded that ethyl maltol [FL-no: 07.047], 3-ethylcyclopentan-1,2-dione [FL-no: 07.057] and the nine structurally related substances [FL-no: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080] can be evaluated through the Procedure.

For maltol [FL-no: 07.014], a micronucleus assay after oral application is required in addition to an *in vivo* Comet assay in order to clarify the genotoxic potential. A combination of the micronucleus assay and the Comet assay in a single study would also be acceptable.The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

Due to the structural similarities and to the lack of data, the remaining substances (including two precursors of a ketone) [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] cannot be evaluated through the Procedure. Additional data on genotoxicity are required for representatives of these 13 substances according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19.

Key words: Alicyclic alpha,beta-unsaturated ketones, flavouring substances, safety evaluation.

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BACKGROUND

Regulation (EC) No 2232/96 of the European Parliament and the Council (EC, 1996) lays down a Procedure for the establishment of a list of flavouring substances, the use of which will be authorised to the exclusion of all other flavouring substances in the EU. In application of that Regulation, a Register of flavouring substances used in or on foodstuffs in the Member States was adopted by Commission Decision 1999/217/EC (EC, 1999a), as last amended by Commission Decision 2008/478/EC (EC, 2008a). Each flavouring substance is attributed a FLAVIS-number (FL-number) and all substances are divided into 34 chemical groups. Substances within a group should have some metabolic and biological behaviour in common.

Substances which are listed in the Register are to be evaluated according to the evaluation programme laid down in Commission Regulation (EC) No 1565/2000 (EC, 2000a), which is broadly based on the Opinion of the Scientific Committee on Food (SCF, 1999). For the submission of data by the manufacturer, deadlines have been established by Commission Regulation (EC) No 622/2002 (EC, 2002b).

After the completion of the evaluation programme the community list of flavouring substances for use in or on foods in the EU shall be adopted (Article 5 (1) of Regulation (EC) No 2232/96) (EC, 1996).

Flavouring Group Evaluation 19 (FGE.19) contains 360 flavouring substances from the EU Register being alpha,beta-unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation (EFSA, 2008b).

The alpha,beta-unsaturated aldehyde and ketone structures were considered by the Panel to be structural alerts for genotoxicity. The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The alpha,beta-unsaturated carbonyls were subdivided into 28 subgroups on the basis of structural similarity (EFSA, 2008b). In an attempt to decide which of the substances could go through the Procedure, a (quantitative) structure-activity relationship (Q)SAR prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI MultiCASE Models and ISS Local Models (Gry et al., 2007)).

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these alpha,beta-unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the Procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni & Netzeva, 2007a; Benigni & Netzeva, 2007b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. The Panel decided that 11 subgroups (1.1.2, 1.1.3, 1.1.4, 2.4, 2.6, 2.7, 3.1, 3.3, 4.1, 4.2 and 4.4) (EFSA, 2008b) should be further examined to determine whether evaluation through the Procedure is feasible. Corresponding to these 11 subgroups 11 Flavouring Group Evaluations (FGEs) were established (FGE.201, 202, 203,

210, 212, 213, 214, 216, 217, 218 and 220). If the Panel concludes for any substances in these 11 FGEs that they cannot be evaluated using the Procedure then it has to be decided if there is a safety concern for certain substances or if additional data are required in order to finalise the evaluation. If the Panel concludes that a genotoxic potential can be ruled out for the substances they will be merged with structurally related substances in other FGEs and evaluated using the Procedure.

TERMS OF REFERENCE

European Food Safety Authority (EFSA) is requested to carry out a risk assessment on flavouring substances prior to their authorisation and inclusion in a community list according to Commission Regulation (EC) No 1565/2000 (EC, 2000a).

ACKNOWLEDGEMENTS

European Food Safety Authority wishes to thank the members of the Working Groups on Flavourings for the preparation of this Opinion: Ulla Beckman Sundh, Vibe Beltoft, Wilfried Bursch, Angelo Carere, Riccardo Crebelli, Karl-Heinz Engel, Henrik Frandsen, Jørn Gry, Rainer Gürtler, Frances Hill, Trine Husøy, John Christian Larsen, Catherine Leclercq, Pia Lund, Wim Mennes, Gerard Mulder, Karin Nørby, Gerard Pascal, Iona Pratt, Gerrit Speijers, Harriet Wallin.

ASSESSMENT

1. Presentation of the Substances in the Flavouring Group Evaluation 213

1.1. Description

The present Flavouring Group Evaluation 213 (FGE.213) concerns 26 substances, which are listed in Table 1.). The 26 substances correspond to subgroup 2.7 of FGE.19 (EFSA, 2008b). Twenty-three of the substances are alpha,beta-unsaturated alicyclic ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 16.044] and three are precursors for such ketones [FL-no: 02.106, 09.305 and 09.525]. Two of these substances [FL-no: 02.106 and 09.305] are precursors of the ketone beta-ionone [FL-no: 07.008] and one [FL-no: 09.525] is a precursor of the ketone maltol [FL-no: 07.014]. Ten of the ketones have the possibility for keto-enol tautomerism [FL-no: 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168]. Based on experimental evidence for other diketones it is anticipated that the enol is the predominant form.

A summary of their current evaluation status by the JECFA is given in Table 2 (JECFA, 1999a; JECFA, 2001b; JECFA, 2006a; JECFA, 2007a).

The alpha,beta-unsaturated aldehyde and ketone structures are considered by the Panel to be structural alerts for genotoxicity (EFSA, 2008b). Accordingly, the available data on genotoxic or carcinogenic activity for the 23 alpha,beta-unsaturated ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 16.044], corresponding to 26 substances in FGE.213, will be considered in this FGE.

The Panel has also taken into consideration the outcome of the predictions from five selected (Q)SAR models (Benigni & Netzeva, 2007a; Gry et al., 2007; Nikolov et al., 2007) on the 23 ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 16.044]. The 23 ketones and their (Q)SAR predictions are shown in Table 3.

2. Toxicity

2.1. (Q)SAR Predictions

In Table 3 the outcomes of the (Q)SAR predictions for possible genotoxic activity in five *in vitro* (Q)SAR models (ISS Local Model-Ames test, DTU-NFI MultiCASE-Ames test, -Chromosomal aberration test in Chinese hamster ovary cells (CHO), -Chromosomal aberration test in Chinese hamster lung cells (CHL), and -Mouse lymphoma test) are presented.

Maltol [FL-no: 07.014], ethyl maltol [FL-no: 07.047] and nootkatone [FL-no: 07.089] were predicted positive with the MultiCASE model on chromosomal aberrations in CHL cells. All other predictions were negative or the substances were out of domain (See Table 3).

2.2. Carcinogenicity Studies

In a combined study of developmental toxicity and carcinogenicity, three successive generations of male and female Charles River CD-COBS rats received 3-ethyl-2-hydroxy-2-cyclopenten-1-one (due to keto-enol tautomerism this substance can exist as two isomers; the keto-isomer is 3-ethylcyclopentan-1,2-dione [FL-no: 07.057], a synonym for the keto-isomer is ethylcyclopentenolone) in the basal diet at doses of 0 (untreated control), 0 (propylene glycol control), 30, 80 or 200 mg/kg body weight (bw) per day. The F_1 generation was initially exposed *in utero*, subsequently via the dams' milk until weaning, and then treated for two years and bred twice (at days 99 and 155). In the F_1 generation, there were 100 animals of each sex in the untreated control group and 50 of each sex in the propylene glycol control and 3-ethyl-2-hydroxy-2-cyclopenten-1-one-treated groups. Survival, clinical symptoms, food consumption, reproductive performance, and haematological and clinical chemical parameters were not adversely affected. Gross pathological and histopathological examination revealed no significant treatment-related effects. The incidence of benign or malignant tumours in treated animals was similar to that in controls. The no observed effect level (NOEL) was 200 mg/kg bw per day (King et al., 1979).

The Panel concluded that 3-ethyl-2-hydroxy-2-cyclopenten-1-one (3-ethylcyclopentan-1,2-dione [FL-no: 07.057]) was not carcinogenic in rats under the study conditions.

Groups of 25 male and female rats were fed for two years on diets containing ethyl maltol [FL-no: 07.047] calculated to deliver 0, 50, 100 and 200 mg ethyl maltol/kg bw/day. No abnormalities were seen as regards survival, clinical appearance, growth rate or food consumption, clinical chemistry, haematology and urinalysis. No histopathological changes and no increases in neoplasms were seen after the treatment with ethyl maltol (Gralla et al., 1969).

Study validation and results are presented in Table 4.

The Panel noted that this study was performed before OECD test guidelines 451/453 (1981) have been established and it does not meet the criteria of these OECD test guidelines with respect to the number of animals. However, the Panel concluded that ethyl maltol was not carcinogenic in rats in this study.

2.3. Genotoxicity Studies

In subgroup 2.7, there are studies available for four substances. For maltol [FL-no: 07.014] eight *in vitro* and three *in vivo* studies have been evaluated. For ethyl maltol [FL-no:07.047] two *in vitro* and one *in vivo* study were evaluated. Numbers of evaluated *in vitro* studies concerning beta-ionone [FL-no: 07.008] and 3-methylcyclopentan-1,2-dione [FL-no: 07.056] were two and one, respectively.

Study validation and results are presented in Table 5 and 6.

In studies which were considered valid, the following results were obtained:

Maltol induced gene mutations in bacteria (Bjeldanes & Chew, 1979) and sister chromatid exchanges (SCE) in human lymphocytes (Jansson et al., 1986). *In vivo*, maltol induced micronuclei in mouse bone marrow after intraperitoneal application (Hayashi et al., 1988). Negative results were obtained in a sex-linked recessive lethal mutation assay in *Drosophila* (Mason et al., 1992). However, the micronucleus assay is considered more relevant than the *Drosophila* assay.

Ethyl maltol induced gene mutations in bacteria (Bjeldanes & Chew, 1979).

A negative result was obtained with beta-ionone in a gene mutation assay in bacteria (Mortelmans et al., 1986).

The validity of other studies was limited or could not be evaluated.

2.4. Conclusion on Genotoxicity and Carcinogenicity

For the substances of this group, the applicabiltity of the (Q)SAR models is very limited since many substances were out of domain in the ISS model and the MultiCASE models.

Two substances [FL-no: 02.106 and 09.305] are precursors of beta-ionone [FL-no: 07.008] and therefore, the conclusions for these two precursors could be based on the conclusions drawn for the corresponding ketone [FL-no: 07.008]. Maltyl isobutyrate [FL-no: 09.525] is a precursor of maltol [FL-no: 07.014], and accordingly, the conclusion for maltyl isobutyrate could be based on the conclusion drawn for maltol.

Maltol and ethyl maltol were considered separately because in contrast to the other substances in this subgroup they contain a ring-oxygen atom.

There is a carcinogenicity study on ethyl maltol [FL-no: 07.047] in rats. Although the number of animals per group were lower than suggested in OECD guidelines they were in accordance with the standards at the time the study was performed and the Panel concluded that the result could overrule the mutagenicity observed with ethyl maltol in bacteria but not the mutagenicity observed with maltol [FL-no: 07.014] *in vitro* and *in vivo*. Since the micronuclei induced by maltol in mice were analysed after intraperitoneal application, a micronucleus assay after oral application is required in addition to an *in vivo* Comet assay in order to clarify the genotoxic potential of maltol. A combination of the micronucleus assay and the Comet assay in a single study would also be acceptable. The result of these assays would also be applicable to maltyl isobutyrate [FL-no: 09.525], which is a precursor of maltol.

No carcinogenicity was observed with 3-ethyl-2-hydroxy-2-cyclopenten-1-one [FL-no: 07.057] in rats. This substance was considered representative for nine substances [FL-no: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080]. Therefore, the Panel concluded that the structural alert for genotoxicity is overruled for 3-ethyl-2-hydroxy-2-cyclopenten-1-one [FL-no: 07.057] as well as for the nine structurally related substances.

For the 13 remaining substances (including two precursors of a ketone) [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] a genotoxic potential could not be ruled out since only one valid negative bacterial genotoxicity study on [FL-no: 07.008] is available for these substances.

3. Conclusions

The Panel concluded that ethyl maltol [FL-no: 07.047], 3-ethylcyclopentan-1,2-dione [FL-no: 07.057] and the nine structurally related substances [FL-no: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080] can be evaluated through the Procedure.

For maltol [FL-no: 07.014], a micronucleus assay after oral application is required in addition to an *in vivo* Comet assay in order to clarify the genotoxic potential. A combination of the micronucleus

assay and the Comet assay in a single study would also be acceptable. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

The remaining 13 substances (including two precursors of a ketone) [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] cannot presently be evaluated through the Procedure. Additional data on genotoxicity are required for representatives of these 13 substances, according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008bb).

TABLE 1: SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 213

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 213

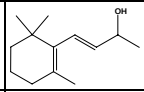
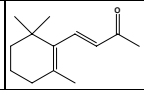
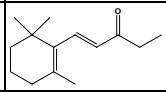
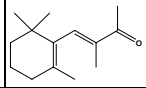
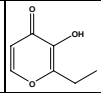
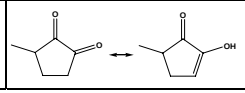
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
02.106 392	4-(2,2,6-Trimethyl-1-cyclohexenyl)but-3-en-2-ol		3625 22029-76-1	Liquid $C_{13}H_{22}O$ 194.32		107 (4 hPa) IR 92 %	1.499 0.927-0.933
07.008 389	beta-Ionone		2595 142 14901-07-6	Liquid $C_{13}H_{20}O$ 192.30	Insoluble 1 ml in 3 ml 70% alcohol	239 IR 95 %	1.517-1.522 0.940-0.947
07.010 399	Methyl-beta- ionone		2712 144 127-43-5	Liquid $C_{14}H_{22}O$ 206.33		238-242 IR 88 %	1.503-1.508 0.930-0.935
07.014 1480	Maltol		2656 148 118-71-8	Solid $C_6H_6O_3$ 126.11	Very slightly soluble Soluble	 159-162 NMR 98 %	n.a. n.a.
07.041	beta-Isomethylionone		 650 79-89-0	Solid $C_{14}H_{22}O$ 206.32	 1 ml in 1 ml	334 62 95 %	n.a. n.a.
07.047 1481	Ethyl maltol		3487 692 4940-11-8	Solid $C_7H_8O_3$ 140.14	Soluble Soluble	 89-93 NMR 99 %	n.a. n.a.
07.056 418	3-Methylcyclopentan-1,2-dione		2700 758 80-71-7	Solid $C_6H_8O_2$ 112.13	1 g in 72 ml water 1 g in 5 ml 90% alcohol	 104-108 IR 95 %	

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 213

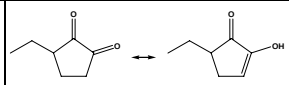
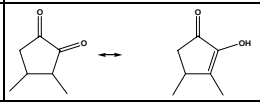
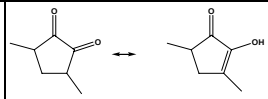
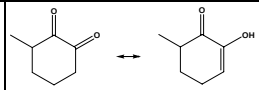
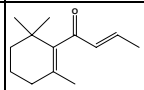
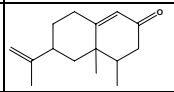
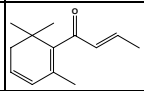
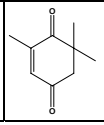
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
07.057 419	3-Ethylcyclopentan-1,2-dione		3152 759 21835-01-8	Solid $C_7H_{10}O_2$ 126.16	Miscible	78-80 (5 hPa) 36-43 IR 90 %	1.47-1.48 (25°) 1.060-1.066
07.075 420	3,4-Dimethylcyclopentan-1,2-dione		3268 2234 13494-06-9	Solid $C_7H_{10}O_2$ 126.16		66 (1 hPa) 68-72 IR 98 %	
07.076 421	3,5-Dimethylcyclopentan-1,2-dione		3269 2235 13494-07-0	Solid $C_7H_{10}O_2$ 126.16	Insoluble	 87-93 MS 98 %	
07.080 425	3-Methylcyclohexan-1,2-dione		3305 2311 3008-43-3	Solid $C_7H_{10}O_2$ 126.16	Insoluble	69-72 (1 hPa) 57-63 IR 98 %	
07.083 384	beta-Damascone		3243 2340 23726-92-3	Liquid $C_{13}H_{20}O$ 192.30	1 ml in 10 ml 95%	67-70 IR 90 %	1.496-1.501 0.934-0.942 (20°)
07.089 1398	Nootkatone		3166 11164 4674-50-4	Liquid $C_{15}H_{22}O$ 218.35	Slightly soluble Soluble	73-103 (1 hPa) NMR 93 %	1.510-1.523 1.003-1.032
07.108 387	beta-Damascenone		3420 11197 23696-85-7	Liquid $C_{13}H_{18}O$ 190.28	1 ml in 10 ml 95% alcohol	60 IR 98 %	1.508-1.514 0.945-0.952 (20°)
07.109 1857	2,6,6-Trimethylcyclohex-2-en-1,4-dione		3421 11200 1125-21-9	Solid $C_9H_{12}O_2$ 152.2	Slightly soluble Soluble	222 23-28 IR NMR 98 %	n.a. n.a.

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 213

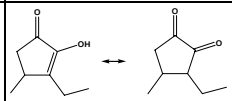
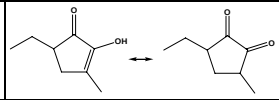
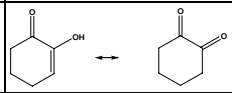
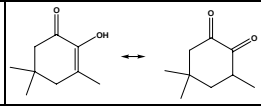
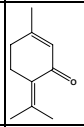
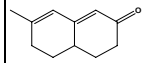
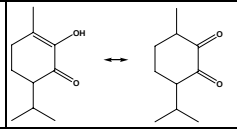
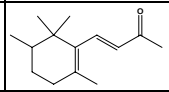
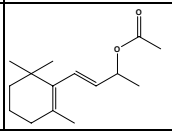
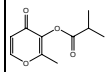
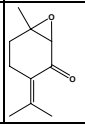
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
07.117 422	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		3453 11077 42348-12-9	Liquid $C_8H_{12}O_2$ 140.18	Slightly insoluble Miscible	 NMR 99 %	1.481-1.487 1.055-1.061
07.118 423	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		3454 11078 53263-58-4	Liquid $C_8H_{12}O_2$ 140.18	Slightly soluble Soluble	 NMR 99 %	1.478-1.484 1.053-1.060
07.119 424	2-Hydroxycyclohex-2-en-1-one		3458 11046 10316-66-2	Solid $C_6H_8O_2$ 112.13	Soluble Soluble	53 (3 hPa) 35-38 IR 99.3 %	
07.120 426	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		3459 11198 4883-60-7	Solid $C_9H_{14}O_2$ 154.21	Slightly soluble Soluble	90-100 (20 hPa) 88 99 %	
07.127 757	p-Mentha-1,4(8)-dien-3-one		3560 11189 491-09-8	Liquid $C_{10}H_{14}O$ 150.22	Insoluble Miscible	233 MS 95 %	1.472-1.478 0.976-0.983
07.136 1405	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		3715 34545-88-5	Solid $C_{11}H_{14}O$ 162.23	Insoluble Soluble	n.a. 36-37 IR 99 %	n.a. n.a.
07.168	2-Hydroxypiperitone 6)		4143 490-03-9	Solid $C_{10}H_{16}O_2$ 168.24	Slightly soluble 1 ml in 1 ml	233 82 NMR MS 98 %	n.a. n.a.

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 213

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one 6)		79-70-9	Liquid $C_{14}H_{22}O$ 206.33	Practically insoluble or insoluble 1 ml in 1 ml	108 (2 hPa) MS 95 %	1.515-1.521 0.943-0.949
09.305 1409	beta-Ionyl acetate 6)		3844 10702 22030-19-9	Liquid $C_{15}H_{24}O_2$ 236.35	Insoluble Soluble	120 (3 hPa) NMR 92 %	1.474-1.484 0.934-0.944
09.525 1482	Maltyl isobutyrate		3462 10739 65416-14-0	Liquid $C_{10}H_{12}O_4$ 196.20	Insoluble Soluble	100 (0.01 hPa) IR 96 %	1.493-1.501 1.140-1.153
16.044 1574	Piperitenone oxide 6)		4199 10508 35178-55-3	Solid $C_{10}H_{14}O_2$ 166.22	Soluble Soluble	 25 NMR MS 95 %	n.a. n.a.

1) Solubility in water, if not otherwise stated.

2) Solubility in 95% ethanol, if not otherwise stated.

3) At 1013.25 hPa, if not otherwise stated.

4) At 20°C, if not otherwise stated.

5) At 25°C, if not otherwise stated.

n.a.: not applicable.

TABLE 2: SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE (BASED ON INTAKES CALCULATED BY THE MSDI APPROACH) (JECFA, 1999A; JECFA, 2001B; JECFA, 2006A; JECFA, 2007A)

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

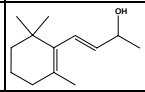
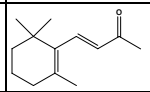
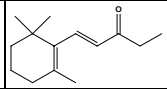
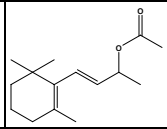
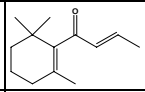
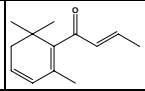
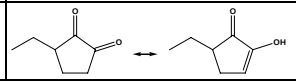
FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) (µg/*capita*/day) EU USA	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]
02.106 392	4-(2,2,6-Trimethyl-1-cyclohexenyl)but-3-en-2-ol		0.73 0.1	Class I A3: Intake below threshold	4)
07.008 389	beta-Ionone		130 100	Class I A3: Intake below threshold	4)
07.010 399	Methyl-beta- ionone		5.4 0.2	Class I A3: Intake below threshold	4)
09.305 1409	beta-Ionyl acetate		ND 9	Class I A3: Intake below threshold	4)
07.083 384	beta-Damascone		37 10	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	4)
07.108 387	beta-Damascenone		73 5	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	4)
07.057 419	3-Ethylcyclopentan-1,2-dione		32 23	Class II A3: Intake below threshold	4)

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

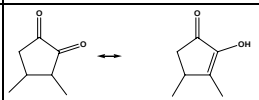
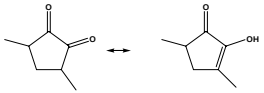
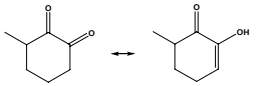
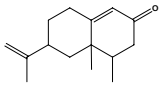
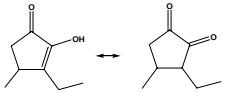
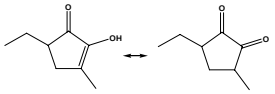
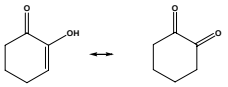
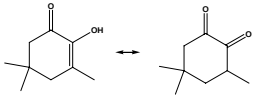
FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) (µg/*capita*/day) EU USA	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]
07.075 420	3,4-Dimethylcyclopentan-1,2-dione		30 2	Class II A3: Intake below threshold	4)
07.076 421	3,5-Dimethylcyclopentan-1,2-dione		35 29	Class II A3: Intake below threshold	4)
07.080 425	3-Methylcyclohexan-1,2-dione		1.3 8	Class II A3: Intake below threshold	4)
07.089 1398	Nootkatone		130 20	Class II A3: Intake below threshold	4)
07.117 422	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		ND 0.17	Class II A3: Intake below threshold	4)
07.118 423	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		ND 0.38	Class II A3: Intake below threshold	4)
07.119 424	2-Hydroxycyclohex-2-en-1-one		0.049 0.76	Class II A3: Intake below threshold	4)
07.120 426	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		1.2 2	Class II A3: Intake below threshold	4)

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

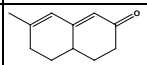
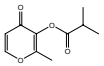
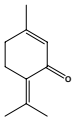
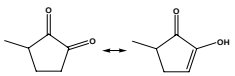
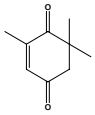
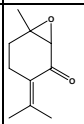
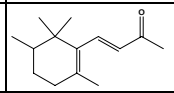
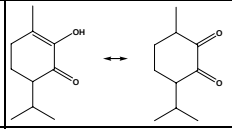
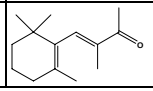
FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) (µg/*capita*/day) EU USA	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]
07.136 1405	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		ND 0.04	Class II A3: Intake below threshold	4)
09.525 1482	Maltyl isobutyrate		20 38	Class II A3: Intake below threshold	4)
07.127 757	p-Mentha-1,4(8)-dien-3-one		0.012 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)
07.014 1480	Maltol		3060 2898	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	4)
07.047 1481	Ethyl maltol		1580 6692	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	4)
07.056 418	3-Methylcyclopentan-1,2-dione		570 710	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	4)
07.109 1857	2,6,6-Trimethylcyclohex-2-en-1,4-dione		50	Class II No evaluation	
16.044 1574	Piperitenone oxide		0.012 0.2	Class III A3: Intake below threshold	4)

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) (µg/*capita*/day) EU USA	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		0.012		Not evaluated by the JECFA.
07.168	2-Hydroxypiperitone		0.0012		Not evaluated by the JECFA.
07.041	beta-Isomethylionone		0.011		Not evaluated by the JECFA.

1) *EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.*

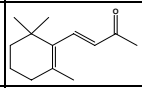
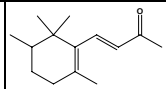
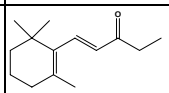
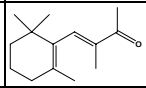
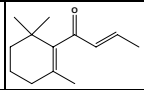
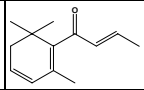
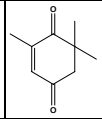
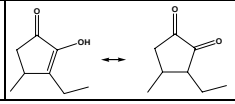
2) *Thresholds of concern: Class I = 1800, Class II = 540, Class III = 90 µg/person/day.*

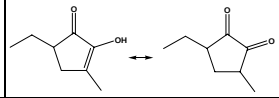
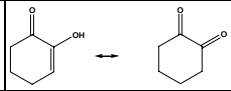
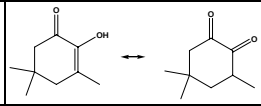
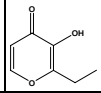
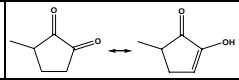
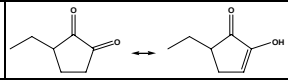
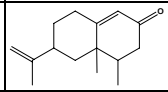
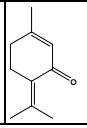
3) *Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.*

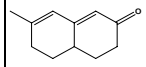
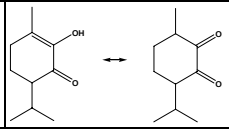
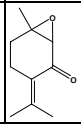
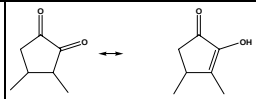
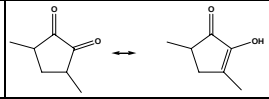
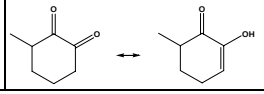
4) *No safety concern based on intake calculated by the MSDI approach of the named compound.*

5) *Data must be available on the substance or closely related substances to perform a safety evaluation.*

TABLE 3: (Q)SAR PREDICTIONS ON MUTAGENICITY IN FIVE MODELS FOR 23 KETONES FROM SUBGROUP 2.7

FL-no JECFA-no	Sub- group	EU Register name	Structural formula	FEMA no CoE no CAS no	ISS Local Model Ames Test TA100	MultiCASE Ames test	MultiCASE Mouse lymphoma test	MultiCASE Chromosomal aberration test in CHO	MultiCASE Chromosomal aberration test in CHL
07.008 389	2.7	beta-Ionone		2595 142 14901-07-6	NEG	NEG	NEG	NEG	EQU
07.200	2.7	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		- - 79-70-9	NEG	NEG	NEG	NEG	EQU
07.010 399	2.7	Methyl-beta- ionone		2712 144 127-43-5	NEG	NEG	OD	OD	EQU
07.041	2.7	beta-Isomethylionone		- 650 79-89-0	NEG	EQU	NEG	NEG	NEG
07.083 384	2.7	beta-Damascone		3243 2340 23726-92-3	OD	NEG	OD	OD	EQU
07.108 387	2.7	beta-Damascenone		3420 11197 23696-85-7	OD	NEG	OD	OD	EQU
07.109	2.7	2,6,6-Trimethylcyclohex-2-en-1,4-dione		3421 11200 1125-21-9	OD	NEG	OD	NEG	EQU
07.117 422	2.7	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		3453 11077 42348-12-9	OD	NEG	NEG	OD	NEG

FL-no JECFA-no	Sub- group	EU Register name	Structural formula	FEMA no CoE no CAS no	ISS Local Model Ames Test TA100	MultiCASE Ames test	MultiCASE Mouse lymphoma test	MultiCASE Chromosomal aberration test in CHO	MultiCASE Chromosomal aberration test in CHL
07.118 423	2.7	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		3454 11078 53263-58-4	OD	NEG	NEG	NEG	NEG
07.119 424	2.7	2-Hydroxycyclohex-2-en-1-one		3458 11046 10316-66-2	OD	OD	NEG	OD	NEG
07.120 426	2.7	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		3459 11198 4883-60-7	OD	NEG	NEG	OD	NEG
07.014 1480	2.7	Maltol		2656 148 118-71-8	OD	OD	NEG	OD	POS
07.047 1481	2.7	Ethyl maltol		3487 692 4940-11-8	OD	OD	NEG	OD	POS
07.056 418	2.7	3-Methylcyclopentan-1,2-dione		2700 758 80-71-7	OD	NEG	NEG	OD	NEG
07.057 419	2.7	3-Ethylcyclopentan-1,2-dione		3152 759 21835-01-8	OD	NEG	NEG	OD	NEG
07.089 1398	2.7	Nootkatone		3166 11164 4674-50-4	OD	NEG	NEG	NEG	POS
07.127 757	2.7	p-Mentha-1,4(8)-dien-3-one		3560 11189 491-09-8	OD	NEG	OD	NEG	NEG

FL-no JECFA-no	Sub- group	EU Register name	Structural formula	FEMA no CoE no CAS no	ISS Local Model Ames Test TA100	MultiCASE Ames test	MultiCASE Mouse lymphoma test	MultiCASE Chromosomal aberration test in CHO	MultiCASE Chromosomal aberration test in CHL
07.136 1405	2.7	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		3715 34545-88-5	OD	NEG	NEG	NEG	OD
07.168	2.7	2-Hydroxypiperitone		4143 490-03-9	OD	NEG	NEG	NEG	NEG
16.044 1574	2.7	Piperitenone oxide		4199 10508 35178-55-3	OD	NEG	OD	OD	OD
07.075 420	2.7	3,4-Dimethylcyclopentan-1,2-dione		3268 2234 13494-06-9	OD	NEG	NEG	OD	NEG
07.076 421	2.7	3,5-Dimethylcyclopentan-1,2-dione		3269 2235 13494-07-0	OD	NEG	NEG	NEG	NEG
07.080 425	2.7	3-Methylcyclohexan-1,2-dione		3305 2311 3008-43-3	OD	NEG	NEG	OD	NEG

Column 2: Structure group 2.7: alpha,beta-unsaturated alicyclic ketones.

Column 6: Local model on aldehydes and ketones, Ames TA100 (NEG: Negative; POS: Positive; OD: Out of domain).

Column 7: MultiCASE Ames test (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

Column 8: MultiCASE Mouse lymphoma test (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

Column 9 MultiCASE Chromosomal aberration in CHO (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

Column 10: MultiCASE Chromosomal aberration in CHL (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

OD, out of applicability domain: not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physicochemical, structural, biological, etc.

TABLE 4: CARCINOGENICITY STUDIES

Table 4: Carcinogenicity Studies

Chemical Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	Results	Reference	Comments[a]
Ethyl maltol [07.047]	Rats; Male , Female 25/sex/group	Diet	0, 50, 100 and 200 mg/kg bw/day	2 years	Males: No increase in tumour incidences. Females: No increase in tumour incidences.	(Gralla et al., 1969)	Valid. The study was performed before the introduction of OECD guidelines but is however considered valid.The NOAEL was 200 mg/kg bw/day, the highest dose tested.
3-Ethylcyclopentan-1,2-dione [07.057]	Rats; Male , Female 50/sex/group	Diet	0, 30, 80 and 200 mg/kg bw/day	2 years	Males: No increase in tumour incidences. Females: No increase in tumour incidences.	(King et al., 1979)	Valid. The study was performed before the introduction of OECD guidelines but is however considered valid.The NOAEL was 200 mg/kg bw/day, the highest dose tested.

a: Validity of carcinogenicity studies:

Valid.

Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).

Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

TABLE 5: GENOTOXICITY (*IN VITRO*)

Table 5: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments [d]
beta-Ionone [07.008]	Gene mutation (preincubation)	*S. typhimurium* TA98, TA100, TA1535, TA1537	1-180 µg/plate	Negative[a]	(Mortelmans et al., 1986)	Valid.
	Gene mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	3 mmol/plate	Negative[a]	(Florin et al., 1980)	Insufficient validity (spot test, not according to OECD guideline, methods and results insufficiently reported).
3-Methylcyclopentan-1,2-dione [07.056]	Reverse mutation	*S. typhimurium* TA1535	10 000 µg/plate	Negative[b]	(Heck et al., 1989)	Validity cannot be evaluated (result not reported in detail)
	Unscheduled DNA synthesis	Rat hepatocytes	500 µg/plate	Negative[b]	(Heck et al., 1989)	Validity cannot be evaluated (result not reported in detail).
Maltol [07.014]	Reverse Mutation	*S. typhimurium* TA100	4.44 µmol/plate (560 µg/plate)	Negative[c]	(Kim et al., 1987b)	Insufficient validity (only one concentration was tested with only one bacterial strain without metabolic activation). The main purpose of the study was to investigate antimutagenic effects.
	Reverse Mutation	*S. typhimurium* TA98 and TA100	Up to 3 mg/plate (3,000 µg/plate)	Positive[a]	(Bjeldanes & Chew, 1979)	Valid.
	Reverse Mutation	*S. typhimurium* TA92, TA98, TA100 and TA104	1.5 to 11 µmol/plate (189 to 1,387 µg/plate)	Negative	(Gava et al., 1989)	Limited validity (data not reported in detail).
	Reverse Mutation	*S. typhimurium* TA1535, TA98, TA100 and TA1537	33 to 10,000 µg/plate	Positive[b]	(Mortelmans et al., 1986)	Valid.
	Reverse Mutation	*S. typhimurium* TA97 and TA102	0.1, 0.5, 1, 5, or 10 mg/plate (100, 500, 1,000, 5,000, or 10,000 µg/plate)	Weak Positive[a]	(Fujita et al., 1992)	Result is considered equivocal. Limited validity (the use of only two strains is not according to OECD guideline).
	DNA Damage (SOS Chromotest)	*Escherichia coli PQ*37	5mM (631 µg/ml)	Negative	(Ohshima et al., 1989)	The test system used is considered inappropriate, due to insufficient validity.
	Sister Chromatid Exchange	Chinese hamster ovary cells	Up to 1.5 µmol/ml (12.6 to 189 µg/ml)	Positive[c]	(Gava et al., 1989)	Validity cannot be evaluated (insufficiently reported: number of cells analysed not reported. Statistical test used not reported). SCEs were reported as SCE per chromosome. Effect was less than two-fold compared to control.
	Sister Chromatid Exchange	Human lymphocytes	Up to 1.0 mM (126.11 µg/ml)	Positive	(Jansson et al., 1986)	Validity cannot be evaluated. Relevance of test system for the evaluation of genotoxicity uncertain.
Ethyl maltol [07.047]	Reverse Mutation	*S. typhimurium* TA 1535, TA1537, TA1538, TA98 and TA100	5 concentrations up to cytotoxicity, or max. 3600 µg/plate	Negative[a]	(Wild et al., 1983)	Limited validity (result not reported in detail, no TA102 or *E. Coli*).
	Reverse Mutation	*S. typhimurium* TA98 and TA100	Up to 2 mg/plate (2,000 µg/plate)	Positive[a]	(Bjeldanes & Chew,	Valid.

Table 5: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments [d]
					1979)	

a: With and without metabolic activation.

b: With metabolic activation.

c: Without metabolic activation.

d: Validity of genotoxicity studies:

Valid.

Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).

Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

TABLE 6: GENOTOXICITY (*IN VIVO*)

Table 6: GENOTOXICITY (*in vivo*)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments [a]
Maltol [07.014]	Micronucleus formation	ddY Mouse bone marrow cells	Intraperitoneal	125, 250, or 500 mg/kg	Positive	(Hayashi et al., 1988)	Valid. The induction of micronuclei was up to about 10-fold compared to control.
	Sex-linked Recessive Lethal Mutation	*D. melanogaster*	Feeding	6,000 ppm (6,000 µg/ml)	Equivocal	(Zimmering et al., 1989)	Limited validity (only one exposure level tested). Test system considered of limited relevance.
	Sex-linked Recessive Lethal Mutation	*D. melanogaster*	Feed	10,000 ppm (10,000 µg/ml)	Negative	(Mason et al., 1992)	Valid, however, test system considered of limited relevance.
	Sex-linked Recessive Lethal Mutation	*D. melanogaster*	Injection	0.2 – 0.3 µl, 10,000 ppm (10,000 µg/ml)	Negative	(Mason et al., 1992)	Valid, however, test system considered of limited relevance.
Ethyl maltol [07.047]	Micronucleus formation	NMRI Mouse bone marrow cells	Intraperitoneal	420, 700, or 980 mg/kg	Negative	(Wild et al., 1983)	Limited validity (injected twice; only analysis at one time point; no PCE/NCE ratio reported).
	Micronucleus formation	NMRI Mouse bone marrow cells	Intraperitoneal	980 mg/kg	Negative	(Wild et al., 1983)	Limited validity (single injection, analysis at three time points, no PCE/NCE ratio reported).
	Sex-linked Recessive Lethal Mutation (Basc test)	*D. melanogaster*	Feed	14, 25 or 50 mM	Negative	(Wild et al., 1983)	Limited validity (limited reporting, test system considered of limited relevance).

a: Validity of genotoxicity studies:

Valid.

Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).

Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

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ABBREVIATIONS

CAS	Chemical Abstract Service
CHL	Chinese hamster lung cell(s)
CHO	Chinese hamster ovary cell(s)
CoE	Council of Europe
DNA	Deoxyribonucleic acid
DTU-NFI	Danish Technical University – National Food Institute
EC	European Commission
EFSA	The European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS	Flavour Information System database
ID	Identity
IR	Infrared spectroscopy
ISS	Istituto Superiore di Sanita
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MS	Mass spectrometry
MSDI	Maximum Survey-derived Daily Intake
NMR	Nuclear magnetic resonance
No	number
NOAEL	No observed adverse effect level
NOEL	No observed effect level
OECD	Organisation for Economic Co-operation and Development
(Q)SAR	(Quantitative) structure-activity relationship
SCF	Scientific Committee on Food
WHO	World Health Organisation

European Food Safety Authority

EFSA Journal 2014; 12(5):3661

SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 213, Revision 1 (FGE.213Rev1): Consideration of genotoxic potential for α,β-Unsaturated Alicyclic ketones and precursors from chemical subgroup 2.7 of FGE.19[1]

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate the genotoxic potential of 26 flavouring substances from subgroup 2.7 of FGE.19 in the Flavouring Group Evaluation 213. In the first version of FGE.213 the Panel concluded based on available genotoxicity data that a concern regarding genotoxicity could be ruled out for [FL-no: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168], but for the remaining 15 substances in subgroup 2.7 further genotoxicity data were required. Based on new submitted genotoxicity data, the Panel concluded in FGE.213Rev1 that the concern regarding genotoxicity could be ruled out for 13 substances in subgroup 2.7 [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] but not for maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525].

KEY WORDS

FGE.213, α,β-Unsaturated alicyclic ketones, flavouring substances, safety evaluation, Subgroup 2.7, FGE.19

[1] On request from the Commission, Question No EFSA-Q-2013-00102 to 00113 and EFSA-Q-2013-00560, EFSA-Q-2013-00561, EFSA-Q-2013-00554, adopted on 10 April 2014.

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† Deceased

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SUMMARY

The Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) is asked to advise the Commission on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Scientific Panel is asked to evaluate flavouring substances using the procedure as referred to in the Commission Regulation EC No 1565/2000.

The present revision of FGE.213, FGE.213Rev1 is due to additional genotoxicity data submitted by the Industry in response to genotoxicity data requests presented in FGE.213. New genotoxicity studies have been submitted for the five substances beta-ionone [FL-no: 07.008], maltol [FL-no: 07.014], beta-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109].

The Flavouring Group Evaluation 213 concerns 26 substances, corresponding to subgroup 2.7 of FGE.19. Twenty-three of the substances are α,β-unsaturated alicyclic ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] and three are precursors for such ketones [FL-no: 02.106, 09.305 and 09.525].

In the first version of FGE.213 the Panel concluded that the genotoxicity concern for ethyl maltol [FL-no: 07.047], 3-ethylcyclopentan-1,2-dione [FL-no: 07.057] and the nine structurally related substances [FL-no: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080] could be ruled out and the 11 substances could accordingly be evaluated through the Procedure.

For maltol [FL-no: 07.014], a micronucleus assay after oral application was required in addition to an *in vivo* Comet assay in order to clarify the genotoxic potential. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

The remaining 13 substances (including two precursors of a ketone) [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] could not be evaluated through the Procedure. Accordingly, additional data on genotoxicity were required for representatives of these 13 substances.

The flavouring Industry has informed that it does not longer support the representative flavouring substance, piperitenone oxide [FL-no: 16.044], for which the Panel requested additional data. Since the previous version of the FGE, one additional substance has been included in subgroup 2.7, tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one [FL-no: 07.224], which is structurally related to the other substances for which the genotoxic potential could not be ruled out.

In FGE.213Rev1 the Panel has evaluated the new data submitted by the Industry in response to the data request presented in FGE.213. Based on these new data the Panel concluded that the genotoxicity concern could be ruled out for the representative substances beta-ionone [FL-no: 07.008], beta-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089], 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] and the nine substances that they represent, being [FL-no: 02.106, 07.010, 07.041, 07.108, 07.127, 07.136, 07.200, 07.224 and 09.305].

The Panel considered also the new data submitted for maltol [FL-no: 07.014] and concluded that for maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525] the concern for genotoxicity could not be ruled out.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The use of flavouring is regulated under Regulation (EC) No 1334/2008[4] of the European Parliament and Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of article 9(a) of this Regulation an evaluation and approval are required for flavouring substances.

The Union List of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012[5]. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000[6].

EFSA concluded that a genotoxic potential of 15 of the α,β-unsaturated alicyclic ketones and precursors in FGE.213 could not be ruled out.

Information on four representative materials have now been submitted by the European Flavour Association. These are beta-ionone [FL-no: 07.008], maltol [FL-no: 07.014], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109].

This information is intended to cover also the re-evaluation of the following eight substances from FGE.19 subgroup 2.7:

- 4-(2,6,6-trimethylcyclohexenyl)but-3-en-2-ol [FL-no: 02.106]
- Methyl-beta-ionone [FL-no: 07.010]
- beta-Isomethylionone [FL-no: 07.041]
- p-Mentha-1,4(8)-dien-3-one [FL-no: 07.127]
- 4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one [FL-no: 07.136]
- 4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one [FL-no: 07.200]
- beta-Ionyl acetate [FL-no: 09.305]
- Maltyl isobutyrate [FL-no: 09.525]

Furthermore, information on one representative material, beta-damascone [FL-no: 07.083] has now been submitted by the European Flavour Association. This information is intended to cover also the re-evaluation of the following two substances.

- beta-Damascenone [FL-no: 07.108]
- trans-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-but-2-en-1-one [FL-no: 07.224]

[4] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34-50

[5] EC (European Commission), 2012. Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p. 1-161

[6] Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. OJ L 180, 19.7.2000, p. 8-16

The Commission asks EFSA to evaluate this new information and depending on the outcome proceed to the full evaluation of the flavouring substances.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests the European Food Safety Authority to carry out a safety assessment on the following 15 flavouring substances: 4-(2,6,6-trimethyl-1-cyclohexenyl)but-3-en-2-ol [FL-no: 02.106], beta-ionone [FL-no: 07.008], methyl-beta-ionone [FL-no: 07.010], maltol [FL-no: 07.014], beta-isomethylionone [FL-no: 07.041], beta-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089], beta-damascenone [FL-no: 07.108], 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109], p-mentha-1,4(8)-dien-3-one [FL-no: 07.127], 4,4a,5,6-tetrahydro-7-methylnapthalen-2(3H)-one [FL-no: 07.136], 4-(2,5,6,6-tetramethyl-1-cyclohexenyl)but-3-en-2-one [FL-no: 07.200], trans-1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-but-2-en-1-one [FL-no: 07.224], beta-ionyl acetate [FL-no: 09.305] and maltyl isobutyrate [FL-no: 09.525] in accordance with Commission Regulation (EC) N° 1565/2000.

HISTORY OF THE EVALUATION OF FGE.19 SUBSTANCES

Flavouring Group Evaluation 19 (FGE.19) contains 360 flavouring substances from the EU Register being α,β-unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation (EFSA, 2008a).

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a). The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The α,β-unsaturated carbonyls were subdivided into subgroups on the basis of structural similarity (EFSA, 2008a). In an attempt to decide which of the substances could go through the Procedure, a (quantitative) structure-activity relationship (Q)SAR prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI-MultiCASE Models and ISS-Local Models, (Gry et al., 2007)).

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these alpha, beta- unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007a; Benigni and Netzeva, 2007b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. Based on these data the Panel decided that 15 subgroups (1.1.1, 1.2.1, 1.2.2, 1.2.3, 2.1, 2.2, 2.3, 2.5, 3.2, 4.3, 4.5, 4.6, 5.1, 5.2 and 5.3) (EFSA, 2008b) could not be evaluated through the Procedure due to concern with respect to genotoxicity. Corresponding to these subgroups, 15 Flavouring Group Evaluations (FGEs) were established: FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225.

For 11 subgroups the Panel decided, based on the available genotoxicity data and (Q)SAR predictions, that a further scrutiny of the data should take place before requesting additional data from the Flavouring Industry on genotoxicity. These subgroups were evaluated in FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220. For the substances in FGE.202, 214 and 218 it was concluded that a genotoxic potential could be ruled out and accordingly these substances will be evaluated using the Procedure. For all or some of the substances in the remaining FGEs, FGE.201, 203, 210, 212, 213, 216, 217 and 220 the genotoxic potential could not be ruled out.

To ease the data retrieval of the large number of structurally related α,β-unsaturated substances in the different subgroups for which additional data are requested, EFSA worked out a list of representative

substances for each subgroup (EFSA, 2008c). Likewise an EFSA genotoxicity expert group has worked out a test strategy to be followed in the data retrieval for these substances (EFSA, 2008b).

The Flavouring Industry has been requested to submit additional genotoxicity data according to the list of representative substances and test strategy for each subgroup.

The Flavouring industry has now submitted additional data and the present FGE concerns the evaluation of these data requested on genotoxicity.

ASSESSMENT

1. HISTORY OF THE EVALUATION OF THE SUBSTANCES BELONGING TO FGE.213

In the EFSA Opinion "List of α,β-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing" (EFSA, 2008c), representative flavouring substances have been selected for FGE.19 subgroup 2.7, corresponding to FGE.213.

In the first scientific opinion on FGE.213 (EFSA, 2009), the Panel concluded that based on the data available the concern with respect to genotoxicity could be ruled out for 11 substances, [FL-no: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 7.117, 07.118, 07.119, 07.120 and 07.168]. Nine of these substances have been evaluated by the JECFA before 2000 to be of no safety concern and in accordance with Commission Regulation (EC) No 1565/2000 no further considerations is requested. The remaining two substances [FL-no: 07.047 and 07.168] will be evaluated in FGE.83Rev1 and FGE.11Rev2, respectively, using the Procedure.

For maltol [FL-no: 07.014], the Panel has requested a combined *in vivo* micronucleus and Comet assay in order to clarify the genotoxic potential. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

For the remaining 13 substances [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] additional data on genotoxicity were required for the representative substances, according to the Opinion of the CEF Panel on the "Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19" (EFSA, 2008b).

FGE	Adopted by EFSA	Link	No. of Substances
FGE.213	27 November 2008	http://www.efsa.europa.eu/en/efsajournal/pub/879.htm	26
FGE.213Rev1	10 April 2014		26

The present FGE.213 Revision 1 (FGE.213Rev1) includes the assessment of additional genotoxicity data submitted by Industry (IOFI, 2012; IOFI, 2013) in reply to a data request presented in FGE.213 (EFSA, 2009).

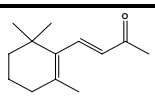
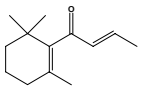
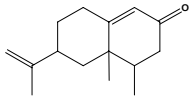
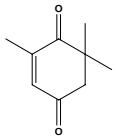
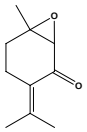
The new data submitted concerns five of the original six representative substances requested by the Panel (EFSA, 2008c), namely beta-ionone [FL-no: 07.008], maltol [FL-no: 07.014], beta-damascone [07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109], (Table 1).

The flavouring Industry has informed that it does not longer support the representative flavouring substance, piperitenone oxide [FL-no: 16.044], for which the Panel requested additional data. However, since piperitenone oxide was a self-representative substance, this will not affect the evaluation of the remaining substances in FGE.213Rev1.

Since the previous version of the FGE, one additional substance has been included in subgroup 2.7, tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one [FL-no: 07.224], which is structurally related to the other substances for which the genotoxic potential could not be ruled out.

The new data submitted for the five representative substances are described and evaluated in Section 4 of the present revision. Sections 2 and 3 report the same information that was present in the earlier version of FGE.213.

Table 1: Representative substances for subgroup 2.7 of FGE.19

FL-no JECFA-no	EU Register name	Structural formula	Comments
07.008 389	beta-Ionone		*In vitro* assays in bacteria and mammalian cells submitted
07.014 1480	Maltol		*In vitro* assays in bacteria and mammalian cells and an *in vivo* combined Comet and micronucleus assay submitted
07.083 384	beta-Damascone		*In vitro* assays in bacteria and mammalian cells and an *in vivo* combined Comet and micronucleus assay submitted
07.089 1398	Nootkatone		*In vitro* assays in bacteria and mammalian cells submitted
07.109 1857	2,6,6-Trimethylcyclohex-2-en 1,4-dione		*In vitro* assays in bacteria and mammalian cells submitted
16.044 1574	Piperitenone oxide		No longer supported by Industry and no data submitted

2. Presentation of the Substances in Flavouring Group Evaluation 213

2.1. Description

The Flavouring Group Evaluation 213 (FGE.213) concerned 26 substances (Table 2), corresponding to subgroup 2.7 of FGE.19. Twenty-three of the substances are α,β-unsaturated alicyclic ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] ([FL-no 16.044] is no longer supported by Industry and one new substance ([FL-no 07.224] has been included in Revision 1) and three are precursors for such ketones [FL-no: 02.106, 09.305 and 09.525]. Two of these substances [FL-no: 02.106 and 09.305] are precursors of the ketone beta-ionone [FL-no: 07.008] and one [FL-no: 09.525] is a precursor of the ketone maltol [FL-no: 07.014]. Ten of the ketones have the possibility for keton-enol tautomerism [FL-no: 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168]. Based on experimental evidence for other diketones it is anticipated that the enol is the predominant form.

Twenty-two of the substances in the present FGE.Rev1 (including the new substance ([FL-no 07.224], excluding ([FL-no 16.044]) have formerly been evaluated by the JECFA (JECFA, 1999; JECFA, 2001; JECFA, 2006a; JECFA, 2006b; JECFA, 2009a), a summary of their current evaluation status by the JECFA is given in Table 3.

As the α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a) the available data on genotoxic or carcinogenic activity for the 26 unsaturated alicyclic ketones and precursor in subgroup 2.7 will be considered in this FGE.

The Panel has also taken into consideration the outcome of the predictions from five selected (Q)SAR models (Benigni & Netzeva, 2007a; Gry et al., 2007; Nikolov et al., 2007) on 22 ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168 and 07.200]. The 22 ketones and their (Q)SAR predictions are shown in Table 4.

3. Toxicity[7]

3.1. (Q)SAR Predictions

In Table 4 the outcomes of the (Q)SAR predictions for possible genotoxic activity in five *in vitro* (Q)SAR models (ISS-Local Model-Ames test, DTU-NFI-MULTICASE-Ames test, Chromosomal aberration test (CHO), Chromosomal aberration test (CHL), and Mouse lymphoma test) are presented.

Maltol [FL-no: 07.014], ethyl maltol [FL-no: 07.047] and nootkatone [FL-no: 07.089] were predicted positive with the MultiCASE model on chromosomal aberrations in CHL cells. All other predictions were negative or the substances were out of domain.

3.2. Genotoxicity Studies

In subgroup 2.7 there are studies available for four substances. For maltol [FL-no: 07.014] eight *in vitro* and three *in vivo* studies have been evaluated. For ethyl maltol [FL-no:07.047] two *in vitro* and one *in vivo* study were evaluated. Numbers of evaluated *in vitro* studies concerning beta-ionone [FL-no: 07.008] and 3-methylcyclopentan-1,2-dione [FL-no: 07.056] were two and one, respectively.

Study validation and results are presented in Tables 5 and 6.

In studies which were considered valid, the following results were obtained:

Maltol induced gene mutations in bacteria (Bjeldanes and Chew, 1979) and sister chromatid exchanges in human lymphocytes (Jansson et al., 1986). *In vivo*, maltol induced micronuclei in mouse bone marrow after intraperitoneal application (Hayashi et al., 1988). Negative results were obtained in a sex-linked recessive lethal mutation assay in Drosophila (Mason et al., 1992). However, the micronucleus assay is considered more relevant than the Drosophila assay.

Ethyl maltol induced gene mutations in bacteria (Bjeldanes and Chew, 1979).

A negative result was obtained with beta-ionone in a gene mutation assay in bacteria (Mortelmans et al., 1986).

The validity of other studies was limited or could not be evaluated.

3.3. Carcinogenicity Studies

In a combined study of developmental toxicity and carcinogenicity, three successive generations of male and female Charles River CD-COBS rats received 3-ethyl-2-hydroxy-2-cyclopenten-1-one (due to keto-enol tautomerism this substance can exist as two isomers; the keto-isomer is 3-ethylcyclopentan-1,2-dione [FL-no: 07.057], a synonym for the keto-isomer is ethylcyclopentenolone) in the basal diet at doses of 0 (untreated control), 0 (propylene glycol control), 30, 80 or 200 mg/kg body weight (bw) per day. The F1 generation was initially exposed in utero, subsequently via the

[7] The data presented in Section 3 is cited from the first version of the present FGE.213. These data are the basis for the conclusions in FGE.213 requesting additional genotoxicity data.

dams' milk until weaning, and then treated for two years and bred twice (at days 99 and 155). In the F1 generation, there were 100 animals of each sex in the untreated control group and 50 of each sex in the propylene glycol control and 3-ethyl-2-hydroxy-2-cyclopenten-1-one-treated groups. Survival, clinical symptoms, food consumption, reproductive performance, and haematological and clinical chemical parameters were not adversely affected. Gross pathological and histopathological examination revealed no significant treatment-related effects. The incidence of benign or malignant tumours in treated animals was similar to that in controls. The No Observed Effect Level (NOEL) was 200 mg/kg bw per day (King et al., 1979).

The Panel concluded that 3-ethyl-2-hydroxy-2-cyclopenten-1-one (3-ethylcyclopentan-1,2-dione [FL-no: 07.057]) was not carcinogenic in rats under the study conditions.

Groups of 25 male and female rats were fed for two years on diets containing ethyl maltol [FL-no: 07.047] calculated to deliver 0, 50, 100 and 200 mg ethyl maltol/kg bw/day. No abnormalities were seen as regards survival, clinical appearance, growth rate or food consumption, clinical chemistry, haematology and urinalysis. No histopathological changes and no increases in neoplasms were seen after the treatment with ethyl maltol (Gralla et al., 1969).

Study validation and results are presented in Table 7.

The Panel noted that this study was performed before OECD test guidelines 451/453 (1981) have been established and it does not meet the criteria of these OECD test guidelines with respect to the number of animals. However, the Panel concluded that ethyl maltol was not carcinogenic in rats in this study.

3.4. Conclusion on Genotoxicity and Carcinogenicity

For the substances of this group, the applicability of the (Q)SAR models is very limited since many substances were out of domain in the ISS model and the MultiCASE models.

Two substances [FL-no: 02.106 and 09.305] are precursors of beta-ionone [FL-no: 07.008] and therefore, the conclusions for these two precursors could be based on the conclusions drawn for the corresponding ketone [FL-no: 07.008]. Maltyl isobutyrate [FL-no: 09.525] is a precursor of maltol [FL-no: 07.014], and accordingly, the conclusion for maltyl isobutyrate could be based on the conclusion drawn for maltol.

Maltol and ethyl maltol were considered separately because in contrast to the other substances in this subgroup they contain a ring-oxygen atom.

There is a carcinogenicity study on ethyl maltol [FL-no: 07.047] in rats. Although the number of animals per group were lower than suggested in OECD guidelines they were in accordance with the standards at the time the study was performed and the Panel concluded that the result could overrule the mutagenicity observed with ethyl maltol in bacteria but not the mutagenicity observed with maltol [FL-no: 07.014] *in vitro* and *in vivo*. Since the micronuclei induced by maltol in mice were analysed after intraperitoneal application, a micronucleus assay after oral application is required in addition to an *in vivo* Comet assay in order to clarify the genotoxic potential of maltol. A combination of the micronucleus assay and the Comet assay in a single study would also be acceptable. The result of these assays would also be applicable to maltyl isobutyrate [FL-no: 09.525] which is a precursor of maltol.

No carcinogenicity was observed with 3-ethyl-2-hydroxy-2-cyclopenten-1-one [FL-no: 07.057] in rats. This substance was considered representative for nine substances [FL-no: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080]. Therefore, the Panel concluded that the structural alert for genotoxicity is overruled for 3-ethyl-2-hydroxy-2-cyclopenten-1-one [FL-no: 07.057] as well as for the nine structurally related substances.

For the 13 remaining substances (including two precursors of a ketone) [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] a genotoxic potential could not be ruled out since only one valid negative bacterial genotoxicity study on [FL-no: 07.008] is available for these substances.

3.5. Conclusions

The Panel concluded that ethyl maltol [FL-no: 07.047], 3-ethylcyclopentan-1,2-dione [FL-no: 07.057] and the nine structurally related substances [FL-no: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080] can be evaluated through the Procedure.

For maltol [FL-no: 07.014], a micronucleus assay after oral application is required in addition to an *in vivo* Comet assay in order to clarify the genotoxic potential. A combination of the micronucleus assay and the Comet assay in a single study would also be acceptable. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

The remaining 13 substances (including two precursors of a ketone) [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] cannot presently be evaluated through the Procedure. Additional data on genotoxicity are requested for representative substances of this subgroup according to the opinion of the Panel on the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008b).

4. Industry Response to Data Requested in FGE.213

4.1. Presentation of the Additional Data

Based on Panel requirements published in FGE.213 (EFSA, 2009), additional data have been provided by the Industry for the representative substances: beta-ionone [FL-no: 07.008], maltol [FL-no: 07.014], beta-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089], and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]

The present FGE.213, Revision 1 (FGE.213Rev1), includes the assessment of these additional genotoxicity data. The study types provided are shown below:

Substance / study type	**Bacterial Mutation**	***In Vitro* Micronucleus**	***In Vivo* Micronucleus combined with Comet**
beta-Ionone [FL-no: 07.008]	(Ballantyne, 2011)	(Stone, 2011a)	
Maltol [FL-no: 07.014]	(Ballantyne, 2012)	(Whitwell, 2012)	(Beevers, 2013a)
beta-Damascone [FL-no: 07.083]	(Bowen, 2011b)	(Stone, 2012)	(Beevers, 2013b; Beevers, 2013c)
Nootkatone [FL-no: 07.089]	(Marzin, 1998)	(Stone, 2011b)	
2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]	(Bowen, 2011a)	(Lloyd, 2011)	

4.2. *In vitro* data

4.2.1. Bacterial Reverse Mutation Assay

beta-Ionone [FL-no: 07.008]

beta-Ionone [FL-no: 07.008] was tested in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Ballantyne, 2011). In the first experiment, the concentrations were 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate of beta-ionone and the plate incorporation methodology was used. Toxicity ranging from slight thinning of the background lawn to complete killing of the tester strains was observed at 1000 and/or 5000 µg/plate for all tester strains in the absence and presence of S9-mix. In the second experiment, the concentrations were 10.24, 25.6, 64, 160, 400 and 1000 µg/plate and the treatments in the presence of S9-mix used the pre-incubation method. Toxicity ranging from thinning of the background lawn and/or reduction in revertant numbers to complete killing of the tester bacteria occurred in all strains at 1000 µg/plate in the absence and presence of S9-mix and was also seen down to 160 and/or 400 µg/plate for some individual strains. The study design complied with current recommendations and an acceptable top concentration was achieved. There was clearly no evidence of any mutagenic effect induced by beta-ionone in any of the strains, either in the absence or presence of S9-mix.

Maltol [FL-no: 07.014]

Maltol [FL-no: 07.014] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Ballantyne, 2012). In the first experiment, the concentrations were 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate of maltol and the plate incorporation methodology was used. Toxicity in the form of reduction of the number of revertants in the tester strain TA102 was observed at 200 µg/plate and above in the presence of S9-mix and 1000 and 5000 µg/plate in the absence of S9-mix. In the second experiment, the concentrations were 51.2, 128, 320, 800, 2000 and 5000 µg/plate and the treatments in the presence of S9-mix used the pre-incubation method in all strains. In tester strain TA102 an additional lower concentration of 20.48 µg/plate was incorporated into the testing protocol in both the absence and presence of S9 to more carefully assess the toxicity observed in Experiment 1. Toxicity in the form of thinning of the background lawn and/or reduction in numbers of revertants occurred at the 5000 µg/plate concentration in strain TA102 in the absence and presence of S9-mix and in strain TA100 only in the presence of S9-mix. The study design complied with current recommendations and an acceptable top concentration was achieved. There was no evidence of any mutagenic effect induced by maltol in any of the strains, either in the absence or presence of S9-mix.

beta-Damascone [FL-no: 07.083]

An Ames assay was conducted in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA102 to assess the mutagenicity of beta-damascone (purity: 95 %), both in the absence and in the presence of metabolic activation by S9-mix, in three separate experiments (Bowen, 2011b). The assay was performed according to OECD Guideline 471 (1997a) and according to GLP principles.

An initial experiment was carried out both in the absence and presence of S9-mix activation in all five strains, using final concentrations of beta-damascone between 0.32 - 5000 µg/plate (0.32, 1.6, 8, 40, 200, 1000, 5000 µg/plate), plus negative (solvent) and positive controls. Evidence of toxicity was observed through thinning of the background lawn to complete killing at 1000 µg/plate and above for strains TA1535, TA1537 and TA102 and/or 5000 µg/plate for strains TA98 and TA100 in the absence and presence of S9-mix. In the second experiment the highest concentration was retained for strains TA98 and TA100 in the absence and presence of S9-mix. In all other tester strains, the highest dose was reduced to 2500 µg/plate based on toxicity observations. In addition, more narrow concentration intervals were used, starting at either 78.13 µg/plate or 156.3 µg/plate (78.13, 156.3, 312.5, 625, 1250, 2500 µg/plate). The standard plate incorporation assay was used in the first experiment but a pre-

incubation step with S9-mix activation treatment was added in the second experiment to increase the chance of detecting a positive response. Evidence of toxicity was observed in TA98 at 625 µg/plate in the presence of S9-mix in addition to strains TA1535, 1537 and TA 102 in the absence and presence of S9-mix, at 1250 µg/plate and above in strain TA98 in the absence of S9-mix and TA100 in the presence of S9-mix and TA100 in the absence of S9-mix at 2500 µg/plate and above.

The third experiment was conducted using strain TA98 in the presence of S9-mix activation using the pre-incubation method. The maximum test concentration was reduced to 1250 µg/plate based on toxicity observed in the previous experiments. In addition, more narrow concentration intervals were used, covering 19.53 to 1250 µg beta-damascone/plate (19.53, 39.06, 78.13, 156.3, 312.5, 625 and 1250 µg/plate). Evidence of toxicity was observed at the highest four concentrations in strain TA98 in the presence of S9-mix. In all three experiments, no statistically significant increases in revertant numbers were observed at any concentration, in any of the strains, either in the presence or absence of S9-mix activation.

The Panel agreed with the conclusion of the study authors that beta-damascone did not induce mutations in five strains of *Salmonella typhimurium*, when tested under the conditions of this study.

Nootkatone [FL-no: 07.089]

Nootkatone [FL-no: 07.089] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence or presence of S9-mix (Marzin, 1998). A preliminary toxicity test to identify appropriate concentrations for the mutagenicity assays was performed in the absence and presence of S9-mix, and cytotoxicity was observed at 50 µg/plate in the absence of S9-mix and at 150 µg/plate in the presence of S9-mix. In the first mutagenicity experiment using plate incorporation methodology the concentrations tested were 0.5, 1.5, 5, 15 and 50 µg/plate in the absence of S9-mix metabolic activation and 1.5, 5, 15, 50 and 150 µg/plate in the presence of S9-mix. In the second experiment the plate incorporation method was used in absence of S9 and the concentrations were 0.5, 1.5, 5, 15 and 50 µg/plate. While the pre-incubation method was used in the presence of S9-mix and the concentrations were and 0.5, 1.5, 5, 15, 50 and 150 µg/plate. Thus, the study design complied with current recommendations and an acceptable top concentration was achieved. There was no evidence of any mutagenic effect induced by nootkatone in any of the strains, either in the absence or presence of S9-mix.

2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]

2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Bowen, 2011a). In the first experiment, the concentrations tested were 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate and plate incorporation methodology was used. In the second experiment, the concentrations were 156.3, 312.5, 625, 1250, 2500 and 5000 µg/plate of 2,6,6-trimethylcyclohex-2-en-1,4-dione and treatments in the presence of S9-mix used the pre-incubation method. The test chemical elicited evidence of cytotoxicity in the form of background lawn thinning or marked reduction of the number of revertants in experiment 1 at 1000 and/or 5000 µg/plate in strains TA102 and TA1535 in the presence of S9-mix and in experiment 2 at 2500 and/or 5000 µg/plate in strain TA102 in the absence and presence of S9-mix. Thus, the study design complied with current recommendations and an acceptable top concentration was achieved. There was no evidence of any mutagenic effect induced by 2,6,6-trimethylcyclohex-2-en-1,4-dione in any of the strains, either in the absence or presence of S9-mix.

Summary of the Bacterial Reverse Mutation Assay for all the substances are reported in Table 8.

4.2.2. Micronucleus Assays

beta-Ionone [FL-no: 07.008]

beta-Ionone [FL-no: 07.008] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat liver S9-mix fraction as an *in vitro* metabolising system. Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells and then treated for 3 hours (followed by 21 hours recovery) with 0, 30, 50 or 60 µg/ml of beta-ionone in the absence of S9-mix and 0, 80, 100 or 120 µg/ml in the presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 52 % and 59 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 5, 15 and 17.50 µg/ml of beta-ionone in the absence of S9-mix with no recovery period. The top concentration induced 58 % cytotoxicity. There were 2 replicate cultures per treatment and 1000 binucleate cells per replicate were scored for micronuclei. Thus, the study design complies with current recommendations (OECD Guideline 487), and acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study. Treatment of cells with beta-ionone for 3 hours with a 21 hours recovery period showed an increase in the frequency of MNBN cells in one single replicate at the concentration of 30 and 120 µg/ml (0.9 % and 1.5 %, respectively) in the absence and presence of S9-mix, respectively. At 30 µg/ml, the lowest concentration tested in the absence of S9-mix, the increase in the frequency of MNBN cells was slightly above the 95 % confidence interval of the historical control range (0.2 - 0.8 %). Also in the presence of S9-mix, one replicate of the lowest concentration tested (80 µg/ml) had an increase in the frequency of MNBN cells at the upper limit of the 95 % confidence interval of the historical control range (0.10 - 1.10 %) but did not reach statistical significance. To ensure that these single occurrences are random an additional 1000 binucleate cells were scored from the concurrent controls, 80 and 120 µg/ml cultures. The scoring of further cells resulted in overall mean frequencies of MNBN cells that were not significantly different from concurrent controls and fell below the upper 95 % confidence interval of the normal control range (recalculated due to change of stain), and therefore showed that the earlier increases were due to chance. It was concluded that beta-ionone [FL-no: 07.008] did not induce micronuclei up to toxic concentrations when assayed in cultured human peripheral lymphocytes for 3 + 21 hours in the absence and presence of S9-mix or when incubated for 24 + 0 hours in the absence of S9-mix (Stone, 2011a).

Maltol [FL-no: 07.014]

Maltol [FL-no: 07.014] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat liver S9-mix fraction as an *in vitro* metabolising system (Whitwell, 2012). Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells and then treated for 3 hours (followed by 21 hours recovery) with 0, 400, 800 or 1262 µg/ml of maltol, the latter being equivalent to 10 mM, in the absence and presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 24 % and 19 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 125, 200 and 300 µg/ml of maltol in the absence of S9-mix with no recovery period. The top concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment, and 1000 binucleate cells per replicate (i.e. 2000 cells per concentration) were scored for micronuclei. Thus, the study design complies with current recommendations (OECD Guideline 487), and acceptable top concentrations, either 10 mM or 50 - 60 % toxicity, were achieved in all parts of the study. A statistically significant increase in the occurrence of MNBN cells was observed following 3 + 21 hours treatment in the presence of S9-mix at the two highest concentrations scored. Statistically significant and concentration-dependent increases in MNBN were seen in the 3 + 21 hours treatment groups in the absence of S9-mix, but it was noted that the increases at the two highest concentrations scored only exceeded historical control ranges in one of the two replicate cultures. No increases were observed in the frequency of MNBN in cells that received continuous (24 + 0 hours) treatment, but due to the cytotoxicity of maltol, lower concentrations were analysed. To further investigate these observations, fluorescence in situ hybridisation (FISH) analysis using human pan-centromeric probes

was conducted to assess whether the mechanism of action could be attributed to chromosome loss (aneuploidy) or chromosome breakage (clastogenicity). Slides were prepared from the two highest concentrations (800 and 1262 µg/ml) in the absence and presence of S9-mix. The FISH analysis revealed that following maltol treatment the majority (69 - 76 %) of micronuclei did not contain a centromere. The Panel concluded that maltol induced micronuclei *in vitro* in cultured human peripheral blood lymphocytes in the presence of rat liver metabolic activation (S9-mix) via a clastogenic mechanism of action (Whitwell, 2012). However, the Panel considered that the results observed in the absence of S9 were equivocal due to the fact that the increases observed (which were statistically significantly different from concurrent solvent control) were not reproduced in replicate cultures.

beta-Damascone [FL-no:07.083]

beta-Damascone (purity: 95 %) was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9 fraction as an *in vitro* metabolising system (Stone, 2012). Cells were stimulated for 48 hours with phytohaemaglutinin to produce exponentially growing cells and then treated for 3 hours (followed by 21 hours recovery) with a large range of concentrations from 2 to 30 µg/ml. For the treatment of 3 hours with a 21 hour recovery period, the concentrations of beta-damascone at 8, 16 and 22 µg/ml or at 12, 16, 18 µg/ml were retained for MN numeration, in the absence or in the presence of S9-mix respectively. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 59 % and 51 %, respectively. Thus, the study design complies with OECD Guideline 487 and follows GLP principles.

In a parallel assay, cells were treated for 24 hours (with no recovery period) in the absence of S9-mix with a large range of concentrations from 1 to 15 µg/ml and the concentrations of 6, 8 and 9 µg/ml of beta-damascone were retained for MN numeration. The top concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment and 1000 binucleate cells per replicate were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487, 2010), and acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study.

Treatment of cells with beta-damascone for 3 + 21 hours in the presence of S9-mix showed a statistically significant concentration-dependent increase in the induction of MNBN cells with 0.55, 2.10 and 2.70 % MNBN cells vs. 0.35 % in the concurrent control and 0.1 to 1.1 % for the historical controls.

Treatment of cells with beta-damascone for 3 + 21 and 24 + 0 hours in the absence of S-9 resulted in sporadic increases in MNBN frequency. These increases were only observed in single replicates and were not concentration related. Therefore, the effect of beta-damascone was further investigated through the scoring of additional cells (2 more replicates of 1000 cells each) from the affected concentrations and concurrent controls.

Treatment of cells, in the absence of S9-mix, for 3 + 21 hours induced a statistically significant increase in the frequency of MNBN cells at 8 and 22 µg/ml (0.80 % and 0.93 %, respectively) compared to concurrent control (0.38 %), but not at the mid dose of 16 µg/ml (0.53 % MNBN cells). The frequency of MNBN cells exceeded the historical controls (0.2 - 0.8 %) in 3 out of 4 replicates at the highest concentration tested (22 µg/ml). Treatment of cells for 24 hours with no recovery period in the absence of S9-mix showed statistically significant increase in the frequency of MNBN cells at the mid dose of 8 µg/ml (0.95 % MNBN cells) when compared to concurrent control (0.40 %) with no correlation to concentration. The frequency of MNBN cells exceeded the historical controls (0 - 1.1 %) only in 1 replicate at 8 µg/ml.

The authors considered that this result reaffirmed the sporadic nature of the induction of MNBN cells in the absence of S9-mix. It was concluded that the treatment with beta-damascone for 3 + 21 hours or

24 + 0 hours (in the absence of S9-mix) induced sporadic increases in MNBN cells when compared to concurrent controls and not concentration related, therefore the results were considered equivocal. In the same test system, beta-damascone did induce micronuclei in cultured human peripheral blood lymphocytes following 3+21 hours treatment in the presence of S9-mix (Stone, 2012). The Panel noted that after the new reading of slides the increase of MNBN cells frequency was still statistically significant even at weak cytotoxic levels.

Therefore, the Panel concluded that beta-damascone is genotoxic in the *in vitro* micronucleus assay on human lymphocytes with metabolic activation and equivocal without metabolic activation.

Nootkatone [FL-no: 07.089]

Nootkatone [FL-no: 07.089] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9-mix fraction as an *in vitro* metabolising system (Stone, 2011b). Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells and then treated for 3 hours (followed by 21 hours recovery) with 0, 50, 70 or 80 µg/ml of nootkatone in the absence of S9-mix and 0, 160, 180 and 185 µg/ml in the presence of S9-mix, respectively. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 60 and 58 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 10, 15, 22 and 24 µg/ml of nootkatone in the absence of S9-mix with no recovery period. The top concentration induced 62 % cytotoxicity. There were 2 replicate cultures per treatment and 1000 binucleate cells per replicate (i.e. 2000 cells per dose) were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487) and acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study. No evidence of chromosomal damage or aneuploidy was observed as frequencies of MNBN cells were not significantly different from concurrent controls and fell within historical control ranges for all treatments with nootkatone in the presence or absence of S9-mix metabolic activation (Stone, 2011b).

2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]

2,6,6-Trimethylcyclohex-2-en-1,4-dione was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9-mix fraction as an *in vitro* metabolising system (Lloyd, 2011). Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells and then treated for 3 hours (followed by 21 hours recovery) with 0, 500, 1000 or 1522 µg/ml of 2,6,6-trimethylcyclohex-2-ene-1,4-dione in the absence of S9-mix and 0, 1000, 1250 and 1522 µg/ml in the presence of S9-mix, respectively, the top concentration being equivalent to 10 mM. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 3 % and 9 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 300, 420 and 550 µg/ml of 2,6,6-trimethylcyclohex-2-ene-1,4-dione in the absence of S9-mix with no recovery period. The top concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment and 1000 binucleate cells per replicate (i.e. 2000 cells per concentration) were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487), and acceptable top concentrations, either 10 mM or 50 - 60 % toxicity, were achieved in all parts of the study. No evidence of chromosomal damage or aneuploidy was observed as frequencies of MNBN cells were not significantly different from concurrent controls and fell within historical ranges for all 2,6,6-trimethylcyclohex-2-ene-1,4-dione treatments in the presence or absence of S9-mix metabolic activation (Lloyd, 2011).

The results of *in vitro* micronucleus studies are summarised in Table 8.

4.3. Genotoxicity *in vivo* data

4.3.1. *In vivo* Combination Assay (Comet + Micronucleus)

Since no positive results were seen in either the bacterial mutation assay or *in vitro* micronucleus tests with beta-ionone [FL-no: 07.008], nootkatone [FL-no: 07.089] or 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109], no *in vivo* follow-up testing was required. To address the effects seen in the *in vitro* micronucleus assay with maltol [FL-no: 07.014] and beta-damascone [FL-no: 07.083] a combination assay comprising of a liver Comet assay and an *in vivo* micronucleus assay in rat, after oral application, was performed to further assess the genotoxic potential for both substances. The results are summarised in Table 9.

Maltol [FL-no: 07.014]

Maltol was evaluated in an *in vivo* micronucleus assay and liver Comet assay in male Han Wistar (HsdHan:WIST) rats, 6 rats per dose group (Beevers, 2013a). The rats were administered 3 doses of 70, 350 and 700 mg/kg bw of maltol by oral gavage at time 0, 24 and 45 hours. Rats were killed and sampled at 48 hours post the initial dose. The maximum tolerable dose was estimated to be 700 mg/kg bw/day based on a range finding study at doses of maltol of 360, 500, 700, 1000, 1500 and 2000 mg/kg bw/day. Clinical observations (piloerection, ataxia, bradypnoea) and mortalities were observed from dose-level of 1000 mg/kg bw/day. For the micronucleus assay 2000 polychromatic erythrocytes (PCE) per rat were scored. The negative control had a normal, low frequency (0.11 %) of micronucleated polychromatic erythrocytes (MNPCE) and a ratio of 53.7 % PCE. The positive control group resulted in a significant increase in MNPCEs (1.58 %) accompanied by some bone marrow toxicity (29.57 % PCE). Although an individual rat in the 700 mg/kg maltol dose group showed a frequency of 9 MNPCE, which resulted in significant heterogeneity in this dose group, this was considered an outlying data point due to the other 5 rats in the group exhibiting normal control level MNPCE frequencies (Beevers, 2013a). Overall, the results showed that there were no statistically significant increases in micronucleus frequency for any dose group after oral treatment with maltol when compared to the vehicle control group. However, in the main experiment, at the dose levels selected, no clinical signs and bone marrow toxicity were observed in any animal in the maltol-treated groups which may reflect the possibility that the bone marrow and liver were not exposed.

In order to clarify this issue, the Panel requested plasma analysis for proof of exposure. Plasma for assay has been obtained from two satellite groups of male animals (3 + 3 animals) dosed with maltol by oral gavage at 700 mg/kg bw/day, during conduction of the main study (Beevers, 2013a). Plasma obtained from 0.5 ml blood drawn from the jugular vein from each animal was frozen in the event that analysis for proof of exposure and toxicokinetics were required. All doses of maltol were given as three administrations, at 0, 24 and 45 hours. A number of three samples of plasma were obtained from one group of animals at 0.5, 2 and 8 hours and three samples from the other group at 1, 4 and 24 hours from the last administration. Analysis of maltol in plasma was performed using a gas chromatography with mass selective detection (GC-MSD) method. From an analytical point of view, the Panel considered the employed approach that was based on the use of ethylmaltol as internal standard as sufficient. Results showed a marked inconsistence between sampling times and animals. In samples collected at 0.5, 2 and 8 hours from last administration maltol was found in 2 out of 3 satellite animals at plasma concentrations of 265 - 283 µg/ml after 0.5 and 2 hours but not longer detectable after 8 hours. In the plasma of the third animal maltol was not detectable at any time. On the other hand, in samples from another animal group (n = 3) collected at 1, 4 and 24 hours from last administration, maltol was found at levels of 75 - 106 µg/l after 1 hour in all 3 animals and not longer detectable after 4 and 24 hours (Mallinson and Hough, 2014). The authors concluded that results obtained provide evidence that maltol is present in plasma shortly after dosing. However, the Panel did not agree with this conclusion and it considered the results of the bioanalytical study as inconclusive.

In the combined Comet assay, liver of rats were removed at 48 hours after the first dose (i.e. 3 hours after the final dose), cut into small pieces and forced through a bolting cloth. Single cell suspensions

were embedded in low melting point agarose on slides and lysed. The DNA was unwound and subjected to electrophoresis at pH > 13 and then neutralised according to standard techniques. For each animal, 100 cells (50 cells/slide from 2 slides) were scored for comets (tail intensity and tail moment) using commercial image analysis equipment.

The Comet assay did not reveal cytotoxicity, necrosis or apoptosis in the hepatocytes as assessed by cloud and halo analysis and the groups treated with maltol showed mean % tail intensities and tail moments that were similar to vehicle controls and fell within historical control ranges. The positive control group treated with ethyl methanesulphonate (EMS) showed significant increases in both parameters (Beevers, 2013a).

Considering that maltol has been shown to induce micronuclei in mouse bone marrow after intraperitoneal injection, the Panel concludes that negative findings observed in the combined bone marrow micronucleus test and Comet assay in the liver of treated rats could not rule out the concern for genotoxicity for maltol since the data provided to prove systemic availability were considered inconclusive due to the inconsistency of the data.

beta-Damascone [FL-no:07.083]

A combined *in vivo* micronucleus assay/liver Comet assay was performed after oral application of beta-damascone (purity: 95.6 %) to further assess the genotoxic potential of beta-damascone and damascones more generally. The results are summarised in Table 9. beta-Damascone was evaluated in an *in vivo* micronucleus assay and liver and duodenum Comet assay in groups of 6 male Han Wistar (HsdHan:WIST) rats per dose group (Beevers, 2013c). Based on a range finding study, 500 mg/kg/day was considered an appropriate estimate of the MTD because the doses of 750 mg/kg/day and above induced moderate to severe clinical signs of toxicity, which included piloerection, decreased activity, hunched posture and abnormal breathing. The rats were administered 3 doses of 125, 250 and 500 mg/kg bw of beta-damascone by oral gavage at time 0, 24 and 45 hours. The rats were sacrificed and sampled at 48 hours post the initial dose.

Animals administered beta-damascone showed clear findings during pathological analysis. Hepatocytes vacuolation was present in animals given 500 mg/kg/day, and was characterised by scattered, occasionally shrunken hepatocytes with perinuclear cytoplasmic eosinophilia and peripheral cytoplasmic vacuolation. Single cell necrosis was present in a single animal given 500 mg/kg/day. Single cell necrosis was characterised by death of individual hepatocytes throughout the liver, with limited inflammatory cell involvement. There was a dose-related reduction in the level of glycogen vacuolation in animals given 250 or 500 mg/kg/day. Glycogen vacuolation was characterised by generally perinuclear, clear, variably sized, indistinctly defined, vacuoles. Finally, increased mitosis was present in animals from all groups given beta-damascone. The greatest severity was present in animals given 250 mg/kg/day, and the lowest incidence was present in animals given 500 mg/kg/day. Increased mitosis was characterised by an increase, above the normal low background incidence, of mitotic figures within the liver parenchyma. Collectively, these findings indicate that the test animals were systemically exposed to beta-damascone.

The negative control had a 0.11 % average rate of micronucleated polychromatic erythrocytes (MNPCE) and a ratio of 50.2 % polychromatic erythrocytes (PCE); 125 mg/kg beta-damascone treatment group had a MNPCE rate of 0.09 % and PCE ratio of 49.17 %; 250 mg/kg treatment group had 0.09 % MNPCE rate and 52.30 % PCE ratio; 500 mg/kg treatment group showed 0.06 % MNPCEs and 37.63 % PCE ratio. The positive control group resulted in 1.54 % MNPCEs and a 43.17 % PCE ratio (Beevers, 2013c). The group mean frequencies observed were similar to concurrent vehicle controls for all dose groups and also were within the historical control values (mean: 0.12 %). There was a reduction in PCE ratio at the highest dose level indicating bone marrow toxicity, which demonstrates target organ exposure. These results showed that there was no statistically significant increase in micronucleus induced with beta-damascone under these test conditions when compared to negative control group. In addition, there were no statistically significant differences among

erythrocyte parameters examined in this study. It was concluded that beta-damascone did not induce micronucleated erythrocytes in rat bone-marrow cells following administration by oral gavage.

The Comet assay in the liver tissue did not reveal cytotoxicity, necrosis or apoptosis in the hepatocytes as assessed by cloud and halo analysis. Hepatocytes of rats dosed with beta-damascone were evaluated for % tail intensities and tail moments (± standard error of the mean, SEM); 125 mg/kg beta-damascone group had 2.45 ± 0.13 % tail intensity and 0.27 ± 0.02 % tail moment; 250 mg/kg group had 2.99 ± 0.31 % tail intensity and 0.33 ± 0.03 tail moment; 500 mg/kg group had 2.93 ± 0.24 % tail intensity and 0.31 ± 0.03 tail moment that were similar to concurrent vehicle controls (tail intensity of 2.67 ± 0.26 % and 0.29 ± 0.03 tail moment) and fell within the testing laboratories historic control range for vehicle controls (0.3 - 8.15 % tail intensity and 0.04 - 0.81 tail moment). The Comet arm of this study confirms that beta-damascone did not induce DNA damage in the liver under the conditions of this study (Beevers, 2013c).

In a satellite study the slides from the duodenum tissue samples collected in the above study (Beevers, 2013c) were analysed for Comet tailing effects (Beevers, 2013b). Duodenum cells of rats dosed with beta-damascone were evaluated for % tail intensities and tail moments (± standard error of the mean, SEM); 125 mg/kg beta-damascone group had 2.01 ± 0.43 % tail intensity and 0.32 ± 0.03 % tail moment; 250 mg/kg group had 1.47 ± 0.15 % tail intensity and 0.16 ± 0.02 tail moment; 500 mg/kg group had 2.03 ± 0.19 % tail intensity and 0.19 ± 0.02 tail moment that were similar to concurrent vehicle controls (tail intensity of 2.24 ± 0.43 % and 0.23 ± 0.04 % tail moment) and fell within the testing laboratories historic control range for vehicle controls (0.3 - 8.15 % tail intensity and 0.04 - 0.81 tail moment). The duodenum Comet arm of this study confirms that beta-damascone did not induce DNA damage in the duodenum under the conditions of this study. The vehicle control data were within historical control ranges (95 % reference range: 0.77 to 8.32 % for tail intensity and 0.08 to 1.15 for tail moment) and the positive control induced a clear increase in DNA damage. The study was therefore confirmed as valid. There was no evidence of duodenum toxicity as would be suggested by increases in clouds or halo cells.

The % tail intensity and tail moment at all dose levels were very similar to the concurrent vehicle control, thus confirming there is no test article-related DNA damage. The additional tissue sample analysis for comet tailing showed a negative result for this study (Beevers, 2013b).

The results from the combined *in vivo* micronucleus induction study and Comet assay show that orally administered beta-damascone did not induce micronucleated erythrocytes in rat bone-marrow cells nor genotoxic events in liver and duodenum of rats.

CONCLUSION

FGE.213 concerned 26 substances, corresponding to subgroup 2.7 of FGE.19 (see Table 1). Twenty-three of the substances are α,β-unsaturated alicyclic ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] and three are precursors for such ketones [FL-no: 02.106, 09.305 and 09.525].

For 11 substances [FL-no: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 7.117, 07.118, 07.119, 07.120 and 07.168] the Panel have ruled out concerns regarding genotoxicity in FGE.213

In the present opinion FGE.213Rev1, new data have been evaluated for the representative of the remaining substances. More specifically, data for beta-ionone [FL-no: 07.008], beta-damascone [FL-no: 07.083], maltol [FL-no: 07.014], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]. All these studies are fully compliant with current guidelines, and stand in contrast to earlier studies previously evaluated in FGE.213.

The combined evidence from *in vitro* and *in vivo* genotoxicity data for the selected representative substances beta-ionone [FL-no: 07.008], beta-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] does not indicate a genotoxic potential. Therefore, these substances and the nine substances that they represent, being [FL-no: 02.106, 07.010, 07.041, 07.108, 07.127, 07.136, 07.200, 07.224 and 09.305] could be evaluated through the procedure.

For maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525], the Panel concluded that the concern for genotoxicity could not be ruled out.

SUMMARY OF SPECIFICATION FOR SUBSTANCES IN FGE.213REV1 (JECFA, 1998; JECFA, 2000; JECFA, 2005A; JECFA, 2005B; JECFA, 2009B)

Table 2: Specification Summary of the Substances in the FGE. 213Rev1

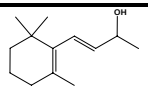
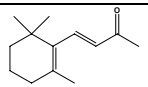
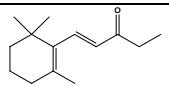
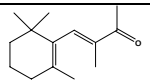
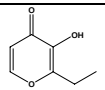
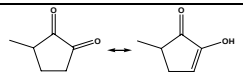
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C [c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
02.106 392	4-(2,2,6-Trimethyl-1-cyclohexenyl)but-3-en-2-ol		3625 22029-76-1	Liquid $C_{13}H_{22}O$ 194.32		107 (4 hPa) IR 92 %	1.499 0.927-0.933
07.008 389	beta-Ionone		2595 142 14901-07-6	Liquid $C_{13}H_{20}O$ 192.30	Insoluble 1 ml in 3 ml 70% alcohol	239 IR 95 %	1.517-1.522 0.940-0.947
07.010 399	Methyl-beta- ionone		2712 144 127-43-5	Liquid $C_{14}H_{22}O$ 206.33		238-242 IR 88 %	1.503-1.508 0.930-0.935
07.014 1480	Maltol		2656 148 118-71-8	Solid $C_6H_6O_3$ 126.11	Very slightly soluble Soluble	159-162 NMR 98 %	n.a. n.a.
07.041	beta-Isomethylionone		4151 650 79-89-0	Solid $C_{14}H_{22}O$ 206.32	Freely soluble	334 62 95 %	n.a. n.a.
07.047 1481	Ethyl maltol		3487 692 4940-11-8	Solid $C_7H_8O_3$ 140.14	Soluble Soluble	89-93 NMR 99 %	n.a. n.a.
07.056 418	3-Methylcyclopentan-1,2-dione		2700 758 80-71-7	Solid $C_6H_8O_2$ 112.13	1 g in 72 ml water 1 g in 5 ml 90% alcohol	104-108 IR 95 %	

Table 2: Specification Summary of the Substances in the FGE. 213Rev1

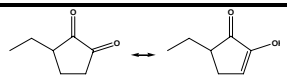
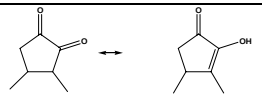
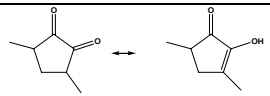
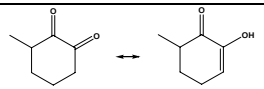
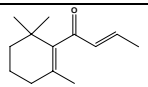
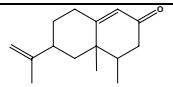
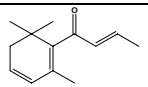
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C [c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
07.057 419	3-Ethylcyclopentan-1,2-dione		3152 759 21835-01-8	Solid $C_7H_{10}O_2$ 126.16	Miscible	78-80 (5 hPa) 36-43 IR 90 %	1.47-1.48 (25°) 1.060-1.066
07.075 420	3,4-Dimethylcyclopentan-1,2-dione		3268 2234 13494-06-9	Solid $C_7H_{10}O_2$ 126.16		66 (1 hPa) 68-72 IR 98 %	
07.076 421	3,5-Dimethylcyclopentan-1,2-dione		3269 2235 13494-07-0	Solid $C_7H_{10}O_2$ 126.16	Insoluble	 87-93 MS 98 %	
07.080 425	3-Methylcyclohexan-1,2-dione		3305 2311 3008-43-3	Solid $C_7H_{10}O_2$ 126.16	Insoluble	69-72 (1 hPa) 57-63 IR 98 %	
07.083 384	beta-Damascone		3243 2340 23726-92-3	Liquid $C_{13}H_{20}O$ 192.30	1 ml in 10 ml 95%	67-70 IR 90 %	1.496-1.501 0.934-0.942 (20°)
07.089 1398	Nootkatone		3166 11164 4674-50-4	Liquid $C_{15}H_{22}O$ 218.35	Slightly soluble Soluble	73-103 (1 hPa) NMR 93 %	1.510-1.523 1.003-1.032
07.108 387	beta-Damascenone		3420 11197 23696-85-7	Liquid $C_{13}H_{18}O$ 190.28	1 ml in 10 ml 95% alcohol	60 IR 98 %	1.508-1.514 0.945-0.952 (20°)

Table 2: Specification Summary of the Substances in the FGE. 213Rev1

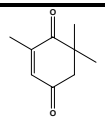
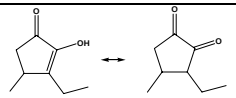
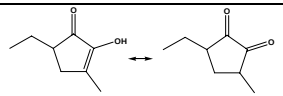
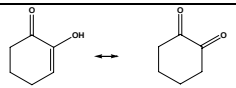
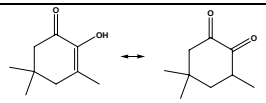
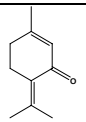
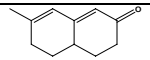
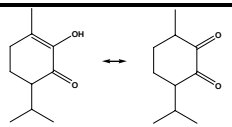
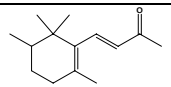
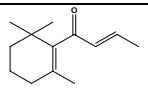
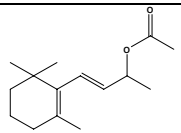
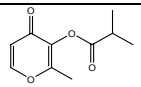
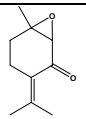
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C [c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
07.109 1857	2,6,6-Trimethylcyclohex-2-en-1,4-dione		3421 11200 1125-21-9	Solid $C_9H_{12}O_2$ 152.2	Slightly soluble Soluble	222 23-28 IR NMR 98 %	n.a. n.a.
07.117 422	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		3453 11077 42348-12-9	Liquid $C_8H_{12}O_2$ 140.18	Slightly insoluble Miscible	 NMR 99 %	1.481-1.487 1.055-1.061
07.118 423	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		3454 11078 53263-58-4	Liquid $C_8H_{12}O_2$ 140.18	Slightly soluble Soluble	 NMR 99 %	1.478-1.484 1.053-1.060
07.119 424	2-Hydroxycyclohex-2-en-1-one		3458 11046 10316-66-2	Solid $C_6H_8O_2$ 112.13	Soluble Soluble	53 (3 hPa) 35-38 IR 99.3 %	
07.120 426	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		3459 11198 4883-60-7	Solid $C_9H_{14}O_2$ 154.21	Slightly soluble Soluble	90-100 (20 hPa) 88 IR 99 %	
07.127 757	p-Mentha-1,4(8)-dien-3-one		3560 11189 491-09-8	Liquid $C_{10}H_{14}O$ 150.22	Insoluble Miscible	233 MS 95 %	1.472-1.478 0.976-0.983
07.136 1405	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		3715 34545-88-5	Solid $C_{11}H_{14}O$ 162.23	Insoluble Soluble	n.a. 36-37 IR 99 %	n.a. n.a.

Table 2: Specification Summary of the Substances in the FGE. 213Rev1

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C [c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
07.168 2038	2-Hydroxypiperitone		4143 490-03-9	Solid $C_{10}H_{16}O_2$ 168.24	Slightly soluble Freely soluble	233 82 NMR MS 98 %	n.a. n.a.
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		 79-70-9	Liquid $C_{14}H_{22}O$ 206.33	Practically insoluble or insoluble Freely soluble	108 (2 hPa) MS 95 %	1.515-1.521 0.943-0.949
07.224	tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one		3243 2340 23726-91-2			 90 %	
09.305 1409	beta-Ionyl acetate		3844 10702 22030-19-9	Liquid $C_{15}H_{24}O_2$ 236.35	Insoluble Soluble	120 (3 hPa) NMR 92 %	1.474-1.484 0.934-0.944
09.525 1482	Maltyl isobutyrate		3462 10739 65416-14-0	Liquid $C_{10}H_{12}O_4$ 196.20	Insoluble Soluble	100 (0.01 hPa) IR 96 %	1.493-1.501 1.140-1.153
16.044 1574	Piperitenone oxide		4199 10508 35178-55-3	Solid $C_{10}H_{14}O_2$ 166.22	Soluble Soluble	 25 NMR MS 95 %	n.a. n.a.

(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95 % ethanol, if not otherwise stated.
(c): At 1013.25 hPa, if not otherwise stated.
(d): At 20°C, if not otherwise stated.
(e): At 25°C, if not otherwise stated.

SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE (JECFA, 1999; JECFA, 2001; JECFA, 2006A; JECFA, 2006B; JECFA, 2009A)

Table 3: Summary of Safety Evaluation Applying the Procedure

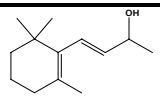
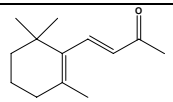
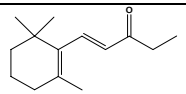
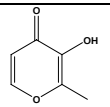
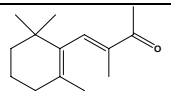
FL-no JECFA-no	EU Register name	Structural formula	MSDI [(a)] (µg/capita/day)	Class [(b)] Evaluation procedure path [(c)]	Outcome on the named compound [(d) or (e)]	EFSA conclusion on the named compound (genotoxicity)
02.106 392	4-(2,2,6-Trimethyl-1-cyclohexenyl)but-3-en-2-ol		0.73 0.1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.008 389	beta-Ionone		130 100	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.010 399	Methyl-beta- ionone		5.4 0.2	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.014 1480	Maltol		3060 2898	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could not be ruled out.Additional data requested.
07.041	beta-Isomethylionone		0.011	Not evaluated by the JECFA		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.12Rev5.

Table 3: Summary of Safety Evaluation Applying the Procedure

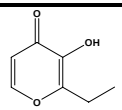
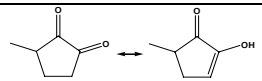
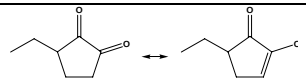
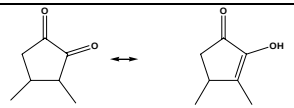
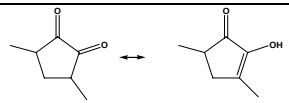
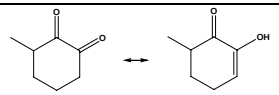
FL-no JECFA-no	EU Register name	Structural formula	MSDI [(a)] (µg/capita/ day)	Class [(b)] Evaluation procedure path [(c)]	Outcome on the named compound [(d) or (e)]	EFSA conclusion on the named compound (genotoxicity)
07.047 1481	Ethyl maltol		1580 6692	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.83Rev1. No safety concern at the estimated level of intake based on the MSDI approach.
07.056 418	3-Methylcyclopentan-1,2-dione		570 710	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.057 419	3-Ethylcyclopentan-1,2-dione		32 23	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.075 420	3,4-Dimethylcyclopentan-1,2-dione		30 2	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.076 421	3,5-Dimethylcyclopentan-1,2-dione		35 29	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.080 425	3-Methylcyclohexan-1,2-dione		1.3 8	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.

Table 3: Summary of Safety Evaluation Applying the Procedure

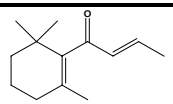
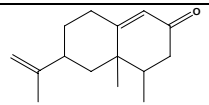
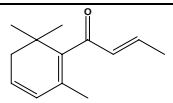
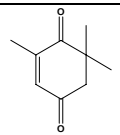
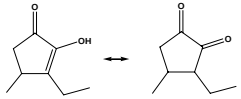
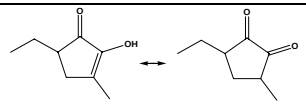
FL-no JECFA-no	EU Register name	Structural formula	MSDI [a] (µg/capita/day)	Class [b] Evaluation procedure path [c]	Outcome on the named compound [d] or [e]	EFSA conclusion on the named compound (genotoxicity)
07.083 384	beta-Damascone		37 10	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.089 1398	Nootkatone		130 20	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.87Rev2.
07.108 387	beta-Damascenone		73 5	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.109 1857	2,6,6-Trimethylcyclohex-2-en-1,4-dione		50	Class II No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.09Rev5.
07.117 422	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		ND 0.17	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.118 423	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		ND 0.38	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.

Table 3: Summary of Safety Evaluation Applying the Procedure

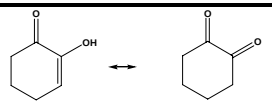
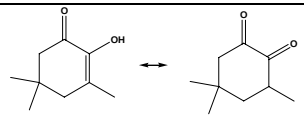
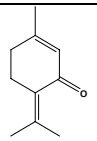
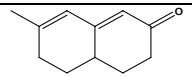
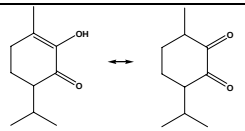
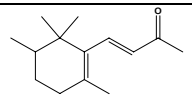
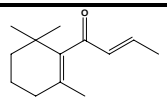
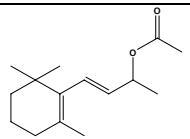
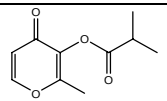
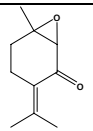
FL-no JECFA-no	EU Register name	Structural formula	MSDI [(a)] (µg/capita/day)	Class [(b)] Evaluation procedure path [(c)]	Outcome on the named compound [(d) or (e)]	EFSA conclusion on the named compound (genotoxicity)
07.119 424	2-Hydroxycyclohex-2-en-1-one		0.049 0.76	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.120 426	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		1.2 2	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.127 757	p-Mentha-1,4(8)-dien-3-one		0.012 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE57Rev1.
07.136 1405	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		ND 0.04	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.87Rev2.
07.168 2038	2-Hydroxypiperitone		0.0012	Class III A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.11Rev2. No safety concern at the estimated level of intake based on the MSDI approach.
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		0.012	Class I No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.12Rev5.

Table 3: Summary of Safety Evaluation Applying the Procedure

FL-no JECFA-no	EU Register name	Structural formula	MSDI [(a)] (µg/capita/day)	Class [(b)] Evaluation procedure path [(c)]	Outcome on the named compound [(d) or (e)]	EFSA conclusion on the named compound (genotoxicity)
07.224	tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one		100	No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.12Rev5.
09.305 1409	beta-Ionyl acetate		ND 9	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.73Rev3. MSDI based on USA production figure.
09.525 1482	Maltyl isobutyrate		20 38	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could not be ruled out.Additional data are requested.
16.044 1574	Piperitenone oxide		0.012 0.2	Class III A3: Intake below threshold	(d)	Evaluated in FGE.213, additional genotoxicity data required. The substance is not supported by the Industy any longer. No further evaluation.

(a): EU MSDI: Amount added to food as flavour in (kg / year) x $10E^9$ / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.
(b): Thresholds of concern: Class I = 1800 µg/person/day, Class II = 540 µg/person/day, Class III = 90 µg/person/day.
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
(d): No safety concern based on intake calculated by the MSDI approach of the named compound.
(e): Data must be available on the substance or closely related substances to perform a safety evaluation.

QSAR PREDICTIONS ON MUTAGENICITY IN FIVE MODELS FOR 22 KETONES FROM SUBGROUP 2.7

Table 4: QSAR Predictions on Mutagenicity for 22 Alicyclic Ketones from Subgroup 2.7

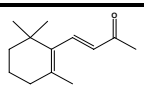
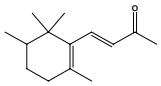
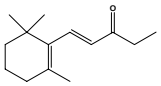
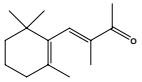
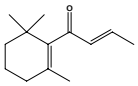
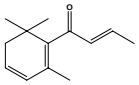
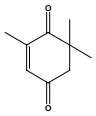
FL-no JECFA-no	EU Register name	Structural formula [a]	ISS Local Model Ames Test TA100 [b]	MultiCASE Ames test [c]	MultiCASE Mouse lymphoma test [d]	MultiCASE Chromosomal aberration test in CHO [e]	MultiCASE Chromosomal aberration test in CHL [f]
07.008 389	beta-Ionone		NEG	NEG	NEG	NEG	EQU
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		NEG	NEG	NEG	NEG	EQU
07.010 399	Methyl-beta- ionone		NEG	NEG	OD	OD	EQU
07.041	beta-Isomethylionone		NEG	EQU	NEG	NEG	NEG
07.083 384	beta-Damascone		OD	NEG	OD	OD	EQU
07.108 387	beta-Damascenone		OD	NEG	OD	OD	EQU
07.109	2,6,6-Trimethylcyclohex-2-en-1,4-dione		OD	NEG	OD	NEG	EQU

Table 4: QSAR Predictions on Mutagenicity for 22 Alicyclic Ketones from Subgroup 2.7

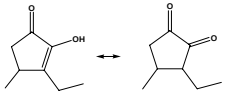
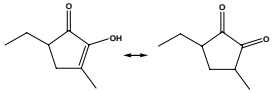
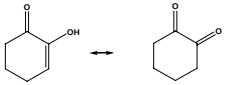
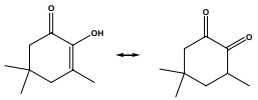
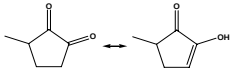
FL-no JECFA-no	EU Register name	Structural formula [a]	ISS Local Model Ames Test TA100 [b]	MultiCASE Ames test [c]	MultiCASE Mouse lymphoma test [d]	MultiCASE Chromosomal aberration test in CHO [e]	MultiCASE Chromosomal aberration test in CHL [f]
07.117 422	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		OD	NEG	NEG	OD	NEG
07.118 423	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		OD	NEG	NEG	NEG	NEG
07.119 424	2-Hydroxycyclohex-2-en-1-one		OD	OD	NEG	OD	NEG
07.120 426	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		OD	NEG	NEG	OD	NEG
07.014 1480	Maltol		OD	OD	NEG	OD	POS
07.047 1481	Ethyl maltol		OD	OD	NEG	OD	POS
07.056 418	3-Methylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG

Table 4: QSAR Predictions on Mutagenicity for 22 Alicyclic Ketones from Subgroup 2.7

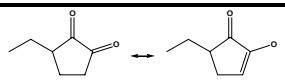
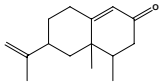
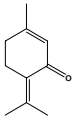
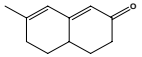
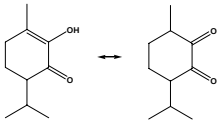
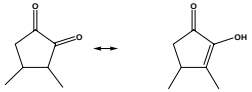
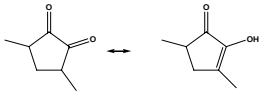
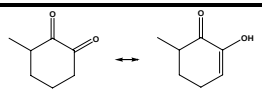
FL-no JECFA-no	EU Register name	Structural formula [a]	ISS Local Model Ames Test TA100 [b]	MultiCASE Ames test [c]	MultiCASE Mouse lymphoma test [d]	MultiCASE Chromosomal aberration test in CHO [e]	MultiCASE Chromosomal aberration test in CHL [f]
07.057 419	3-Ethylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG
07.089 1398	Nootkatone		OD	NEG	NEG	NEG	POS
07.127 757	p-Mentha-1,4(8)-dien-3-one		OD	NEG	OD	NEG	NEG
07.136 1405	4,4a,5,6-Tetrahydro-7-methylnapthalen-2(3H)-one		OD	NEG	NEG	NEG	OD
07.168 -	2-Hydroxypiperitone		OD	NEG	NEG	NEG	NEG
07.075 420	3,4-Dimethylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG
07.076 421	3,5-Dimethylcyclopentan-1,2-dione		OD	NEG	NEG	NEG	NEG

Table 4: QSAR Predictions on Mutagenicity for 22 Alicyclic Ketones from Subgroup 2.7

FL-no JECFA-no	EU Register name	Structural formula [(a)]	ISS Local Model Ames Test TA100 [(b)]	MultiCASE Ames test [(c)]	MultiCASE Mouse lymphoma test [(d)]	MultiCASE Chromosomal aberration test in CHO [(e)]	MultiCASE Chromosomal aberration test in CHL [(f)]
07.080 425	3-Methylcyclohexan-1,2-dione		OD	NEG	NEG	OD	NEG

(a): Structure group 2.7: α,β-unsaturated ketones.
(b): Local model on aldehydes and ketones, Ames TA100. (NEG: Negative; POS: Positive; OD*: out of domain).
(c): MultiCase Ames test (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(d): MultiCase Mouse Lymphona test (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(e): MultiCase Chromosomal aberration in CHO (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(f): MultiCase Chromosomal aberration in CHL (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
* OD, out of applicability domain: not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physicochemical, structural, biological etc.

GENOTOXICITY DATA (*IN VITRO*) CONSIDERED BY THE PANEL IN FGE.213

Table 5: Genotoxicity (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments [(d)]
beta-Ionone [07.008]	Gene mutation(preincubation)	*S. typhimurium* TA98, TA100, TA1535, TA1537	1-180 µg/plate	Negative [(a)]	(Mortelmans et al., 1986)	Valid.
	Gene mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	3 mmol/plate	Negative [(a)]	(Florin et al., 1980)	Insufficient validity (spot test, not according to OECD guideline, methods and results insufficiently reported).
3-Methylcyclopentan-1,2-dione [07.056]	Reverse mutation	*S. typhimurium* TA1535	10 000 µg/plate	Negative [(b)]	(Heck et al., 1989)	Validity cannot be evaluated (result not reported in detail).
	Unscheduled DNA synthesis	Rat hepatocytes	500 µg/plate	Negative [(b)]	(Heck et al., 1989)	Validity cannot be evaluated (result not reported in detail).
Maltol [07.014]	Reverse Mutation	*S. typhimurium* TA100	4.44 µmol/plate (560 µg/plate)	Negative [(c)]	(Kim et al., 1987)	Insufficient validity (only one concentration was tested with only one bacterial strain without metabolic activation). The main purpose of the study was to investigate antimutagenic effects.
	Reverse Mutation	*S. typhimurium* TA98 and TA100	Up to 3 mg/plate (3000 µg/plate)	Positive [(a)]	(Bjeldanes and Chew, 1979)	Valid.
	Reverse Mutation	*S. typhimurium* TA92, TA98, TA100 and TA104	1.5 to 11 µmol/plate (189 to 1387 µg/plate)	Negative	(Gava et al., 1989)	Limited validity (data not reported in detail).
	Reverse Mutation	*S. typhimurium* TA1535, TA98, TA100 and TA1537	33 to 10 000 µg/plate	Positive [(b)]	(Mortelmans et al., 1986)	Valid.

	Reverse Mutation	*S. typhimurium* TA97 and TA102	0.1, 0.5, 1, 5, or 10 mg/plate (100, 500, 1000, 5000, or 10 000 µg/plate)	Weak Positive [(a)]	(Fujita et al., 1992)	Result is considered equivocal. Limited validity (the use of only two strains is not according to OECD guideline).
	DNA Damage (SOS Chromotest)	*Escherichia coli PQ*37	5 mM (631 µg/ml)	Negative	(Ohshima et al., 1989)	The test system used is considered inappropriate, due to insufficient validity.
	Sister Chromatid Exchange	Chinese hamster ovary cells	Up to 1.5 µmol/ml (12.6 to 189 µg/ml)	Positive [(c)]	(Gava et al., 1989)	Validity cannot be evaluated (insufficiently reported: number of cells analysed not reported. Statistical test used not reported). SCEs were reported as SCE per chromosome. Effect was less than twofold compared to control.
	Sister Chromatid Exchange	Human lymphocytes	Up to 1.0 mM (126.11 µg/ml)	Positive	(Jansson et al., 1986)	Validity cannot be evaluated. Relevance of test system for the evaluation of genotoxicity uncertain.
Ethyl maltol [07.047]	Reverse Mutation	*S. typhimurium* TA 1535, TA1537, TA1538, TA98 and TA100	5 concentrations up to cytotoxicity, or max. 3600 µg/plate	Negative [(a)]	(Wild et al., 1983)	Limited validity (result not reported in details, no TA102 or *E. Coli*).
	Reverse Mutation	*S. typhimurium* TA98 and TA100	Up to 2 mg/plate (2000 µg/plate)	Positive [(a)]	(Bjeldanes and Chew, 1979)	Valid.

(a): With and without metabolic activation
(b): With metabolic activation
(c): Without metabolic activation
(d): Validity of genotoxicity studies:
Valid
Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation)
Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system)
Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

GENOTOXICITY DATA (*IN VIVO*) CONSIDERED BY THE PANEL IN FGE.213

Table 6: Genotoxicity (*in vivo*)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments [(a)]
Maltol [07.014]	Micronucleus formation	ddY Mouse bone marrow cells	Intraperitoneal	125, 250, or 500 mg/kg	Positive	(Hayashi et al., 1988)	Valid. The induction of micronuclei was up to about 10-fold compared to control.
	Sex-linked Recessive Lethal Mutation	*Drosophila melanogaster*	Feeding	6000 ppm (6000 µg/ml)	Equivocal	(Zimmering et al., 1989)	Limited validity (only one exposure level tested). Test system considered of limited relevance.
	Sex-linked Recessive Lethal Mutation	*Drosophila melanogaster*	Feed	10 000 ppm (10 000 µg/ml)	Negative	(Mason et al., 1992)	Valid, however, test system considered of limited relevance.
	Sex-linked Recessive Lethal Mutation	*Drosophila melanogaster*	Injection	0.2 – 0.3 µl, 10 000 ppm (10 000 µg/ml)	Negative	(Mason et al., 1992)	Valid, however, test system considered of limited relevance.
Ethyl maltol [07.047]	Micronucleus formation	NMRI Mouse bone marrow cells	Intraperitoneal	420, 700, or 980 mg/kg	Negative	(Wild et al., 1983)	Limited validity (injected twice; only analysis at one time point; no PCE/NCE ratio reported).
	Micronucleus formation	NMRI Mouse bone marrow cells	Intraperitoneal.	980 mg/kg	Negative	(Wild et al., 1983)	Limited validity (single injection, analysis at three time points, no PCE/NCE ratio reported).
	Sex-linked Recessive Lethal Mutation (Basc test)	*Drosophila melanogaster*	Feed	14, 25 or 50 mM	Negative	(Wild et al., 1983)	Limited validity (limited reporting, test system considered of limited relevance).

(a): Validity of genotoxicity studies:
Valid.
Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).
Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).
Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

CARCINOGENICITY STUDIES CONSIDERED BY THE PANEL IN FGE.213

Table 7: Carcinogenicity Studies

Chemical Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	Results	Reference	Comments [(a)]
Ethyl maltol [07.047]	Rats; Male, Female 25/sex/group	Diet	0, 50, 100 and 200 mg/kg bw/day	2 years	Males: No increase in tumour incidences Females: No increase in tumour incidences	(Gralla et al., 1969)	Valid. The study was performed before the introduction of OECD guidelines but is however considered valid.The NOAEL was 200 mg/kg bw/day, the highest dose tested.
3-Ethylcyclopentan-1,2-dione [07.057]	Rats; Male, Female 50/sex/group	Diet	0, 30, 80 and 200 mg/kg bw/day	2 years	Males: No increase in tumour incidences Females: No increase in tumour incidences	(King et al., 1979a)	Valid. The study was performed before the introduction of OECD guidelines but is however considered valid.The NOAEL was 200 mg/kg bw/day, the highest dose tested.

(a): Validity of genotoxicity studies:
Valid.
Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).
Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).
Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

GENOTOXICITY DATA (*IN VITRO*) CONSIDERED BY THE PANEL IN FGE.213REV1

Table 8: Summary of Additional *in vitro* Genotoxicity Data for FGE.213Rev1

Chemical Name [FL-no:]	Test System *in vitro*	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
beta-Ionone [07.008]	Reverse Mutation	*S. typhimurium* TA98, TA100, TA102, TA1535 and TA1537	0.32-5000 µg/plate [1,2]	Negative	Ballantyne, 2011	Evidence of toxicity was observed in all strains at 1000 µg/plate and above in the absence and in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA98, TA100, TA102, TA1535 and TA1537	10.24-1000 µg/plate [2,4] or [3,5]	Negative		Evidence of toxicity was observed in all strains at 1000 µg/plate in the absence and presence of S9-mix, and in most cases these toxic effects also extended down to concentrations of 160 or 400 µg/plate. Study design complied with current recommendations.
	Micronucleus Assay	Human peripheral blood lymphocytes	30-60 µg/ml [4,6] 80-120 µg/ml [5,6] 5-17.5 µg/ml [4,7]	Negative	Stone, 2011a	The top concentrations induced 50-60 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.
Maltol [07.014]	Reverse Mutation	*S. typhimurium* TA98, TA100 and TA102, TA1535 and TA1537	0.32-5000 µg/plate [1,2]	Negative	Ballantyne, 2012	Evidence of toxicity was observed in TA102 at 1000 and 5000 µg/plate in the absence of S9-mix and at 200 µg/plate and above in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. typhimurium TA98, TA100, TA1535 and TA1537	51.2-5000 µg/plate [2,4] or [3,5]	Negative		Toxicity was observed at 5000 µg/plate in strain TA100 only in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.

Table 8: Summary of Additional *in vitro* Genotoxicity Data for FGE.213Rev1

Chemical Name [FL-no:]	Test System *in vitro*	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
		S. typhimurium TA102	20.48-5000 µg/plate [2,4] or [3,5]	Negative		Evidence of toxicity was observed at 5000 µg/plate in the absence and presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus Assay	Human peripheral blood lymphocytes	400-1262 µg/ml [4,6] 400-1262 µg/ml [5,6] 125-300 µg/ml [4,7]	Equivocal Positive Negative	Whitwell, 2012	The top concentrations in the 3+21 hours treatments in the absence and presence of S9-mix induced, respectively, 24% and 19% of toxicity. The top concentration in the 24+0 hours treatment in the absence of S9-mix induced 57 % toxicity. There was evidence of micronuclei induction when tested for 3+21 hours in the presence of S9-mix, while in absence of S9-mix the data were considered equivocal. However, no induction of micronuclei was observed in the continuous exposure test. Study design complies with OECD Guideline 487.
beta-Damascone [07.083]	Reverse Mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	0.32-5000 µg/plate [1,2]	Negative	Bowen, 2011b	Toxicity was observed at 1000 and/or 5000 µg/plate across all strains in the absence and presence of S-9; no clear evidence of toxicity in TA100 in the presence of S9-mix. No statistically significant increase in revertant numbers was seen at any concentration, either in the presence or absence of S9-mix.
		S. typhimurium , TA1535, TA1537 and TA102 *S. typhimurium* TA98, TA100	78.13-2500 µg/plate [2,4] or [3,5] 156.3-5000 µg/plate [2,4] or [3,5]	Negative Negative		Evidence of toxicity was observed at the highest three or four concentrations across all strains in the absence and presence of S9-mix. No statistically significant increase in revertant numbers was seen at any concentration, either in the presence or absence of S9-mix
		S. typhimurium TA98	19.3-1250 µg/plate [3,5]	Negative		Evidence of toxicity was observed at the highest four concentrations in strain TA98 in the presence of S9-mix. No statistically significant increase in revertant numbers was seen at any

Table 8: Summary of Additional *in vitro* Genotoxicity Data for FGE.213Rev1

Chemical Name [FL-no:]	Test System *in vitro*	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
						concentration, in the presence of S9-mix.
	Micronucleus Assay	Human peripheral blood lymphocytes	8-22 µg/ml [6,4] 12-18 µg/ml[6,5] 6-9 µg/ml [7,4]	Equivocal [6,4] Positive [6,5] Equivocal [7,4]	Stone, 2012	Positive result was obtained in the 3+21 hour treatment in the presence of S9-mix. Study design complies with OECD Guideline 487.
Nootkatone [07.089]	Reverse Mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	0.5-50 µg/plate [2,4] 1.5.5-150 µg/plate [2,5] 0.5-50 µg/plate [2,4] 0.5-150 µg/plate [3,5]	Negative	Marzin, 1998	Evidence of toxicity was observed at 50 µg/plate in all strains in the absence of S9-mix and at 150 µg/plate in all strains in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus Assay	Human peripheral blood lymphocytes	50-80 µg/ml [4,6] 160-185 µg/ml [5,6] 10-24 µg/ml [4,7]	Negative	Stone, 2011b	The top concentrations in all parts of the study induced > 50 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.
2,6,6-Trimethylcyclohex-2-en-1,4-dione [07.109]	Reverse Mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	0.32-5000 µg/plate [1,2]	Negative	Bowen, 2011a	Evidence of toxicity was observed at 1000 and /or 5000 µg/plate in strains TA102 and TA1535 in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
			156.3-5000 µg/plate [2,4] or [3,5]	Negative		Evidence of toxicity was observed in TA102 at 2500 and 5000 µg/plate. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus Assay	Human peripheral blood lymphocytes	500-1522 µg/ml [4,6] 1000-1522 µg/ml [5,6] 300-550 µg/ml [4,7]	Negative	Lloyd, 2011	The top concentrations in the 3+21 hours in the absence and presence of S9-mix were 10 mM. The top concentration in the 24+0 hours in the absence of S9-mix induced 57 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.

[1] With and without S-9 metabolic activation; [2] Plate incorporation method; [3] Pre-incubation method; [4] Without S-9 metabolic activation; [5] With S-9 metabolic activation; [6] 3-hour incubation with 21-hour recovery period; [7] 24-hour incubation with no recovery period

GENOTOXICITY DATA (*IN VIVO*) CONSIDERED BY THE PANEL IN FGE.213REV1

Table 9: Summary of Additional *in vivo* Genotoxicity Data Submitted for FGE.213Rev1

Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments
Maltol [07.014]	Micronucleus assay	Han Wistar Rat; M	Gavage	70, 350, 700 mg/kg bw/day [1]	Inconclusive	Beevers, 2013a	The average MNPCE appearance frequency and ratio of PCE at all dose levels fell within concurrent and historical control ranges. However, evidence of animal exposure was inconclusive. The study was performed in compliance with OECD Guideline 474.
	Comet assay	Han Wistar Rat; M	Gavage		Negative		Mean % tail intensity and mean tail moment were within historical control range at all test doses. The study was performed in compliance with recommendations of the Comet and IWGT workshop, Japanese Center for the Validation of Alternative Methods (JaCVAM) and current literature.
beta-Damascone [07.083]	Micronucleus assay	Han Wistar Rat; M	Gavage	125, 250 and 500 mg/kg bw/day [1]	Negative	Beevers, 2013b,c	The average MNPCE appearance frequency and ratio of PCE at all dose levels fell within concurrent and historical control ranges. The study was performed in compliance with OECD Guideline 474.
	Comet assay	Han Wistar Rat; M	Gavage		Negative		Mean % tail intensity and mean tail moment were within historical control range at all test doses. The study was performed in compliance with recommendations of the Comet and IWGT workshop, Japanese Center for the Validation of Alternative Methods (JaCVAM) and current literature.

[1] Administered via gavage in 3 doses at times 0, 24 and 45 hours with sacrifice and harvest at 48 hours

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ABBREVIATIONS

BW	Body Weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHO	Chinese Hamster Ovary (cells)
CHL	Chinese Hamster Lung (cells)
CoE	Council of Europe
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
FGE	Flavouring Group Evaluation
FISH	Fluorescence In Situ Hybridisation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
ID	Identity
IOFI	International Organization of the Flavor Industry
i.p.	intraperitoneal
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MNBN	MicroNucleated BiNucleate cells
MNPCE	Micronucleated Polychromatic Erythrocytes
MS	Mass Spectrometry
MSDI	Maximised Survey-derived Daily Intake
MTD	Maximum Tolerated Dose
NCE	NormoChromatic Erythrocytes
NMR	Nuclear Magnetic Resonance
No	Number
NOEL	No Observed Effect Level
NAOEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
PCE	PolyChromatic Erythrocytes
(Q)SAR	(Quantitative) Structure Activity Relationship
RI	Replication Index
SCF	Scientific Committee on Food
WHO	World Health Organisation

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Effects of Flavoring and Casing Ingredients on the Toxicity of Mainstream Cigarette Smoke in Rats

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A series of in vitro and in vivo studies evaluated the potential effects of tobacco flavoring and casing ingredients. Study 1 utilized as a reference control cigarette a typical commercial tobacco blend without flavoring ingredients, and a test cigarette containing a mixture of 165 low-use flavoring ingredients. Study 2 utilized the same reference control cigarette as used in study 1 and a test cigarette containing eight high-use ingredients. The in vitro Ames *Salmonella typhimurium* assay did not show any increase in mutagenicity of smoke condensate from test cigarettes designed for studies 1 and 2 as compared to the reference. Sprague-Dawley rats were exposed by nose-only inhalation for 1 h/day, 5 days/wk for 13 wk to smoke from the test or reference cigarettes already described, or to air only, and necropsied after 13 wk of exposure or following 13 wk of recovery from smoke exposure. Exposure to smoke from reference or test cigarettes in both studies induced increases in blood carboxyhemoglobin (COHb) and plasma nicotine, decreases in minute volume, differences in body or organ weights compared to air controls, and a concentration-related hyperplasia, squamous metaplasia, and inflammation in the respiratory tract. All these effects were greatly decreased or absent following the recovery period. Comparison of rats exposed to similar concentrations of test and reference cigarette smoke indicated no difference at any concentration. In summary, the results did not indicate any consistent differences in toxicologic effects between smoke from cigarettes containing the flavoring or casing ingredients and reference cigarettes.

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Flavoring ingredients are added to tobacco during the manufacture of many types of commercial cigarettes, and humectants such as glycerol are added to increase the moisture-holding capacity of the tobacco. There has been much speculation about the effect of these added ingredients on the toxicity of the resultant smoke. Wynder and Hoffman (1967) hypothesized that adding

nontobacco ingredients might increase or decrease the toxic effects of inhaled tobacco smoke, and later publications (LaVoie et al., 1980; Hoffman and Hoffman, 1997, 2001; World Health Organization, 2001) supported that hypothesis. Recently published research results (Gaworski et al., 1998; Paschke et al., 2002; Rodgman, 2002a, 2002b; Rodgman and Green, 2002; Carmines, 2002; Rustemeier et al., 2002; Roemer et al., 2002; Vanscheeuwijck et al., 2002; Baker et al., 2004) have presented data from in vitro, and in vivo toxicity studies that indicate the addition of ingredients to tobacco does not increase the toxicity of the smoke. Baker et al. (2004), using a pyrolysis technique that mimics closely the combustion conditions inside burning cigarettes (Baker and Bishop, 2004), studied the effects of pyrolysis on the chemistry, in vitro genotoxicity and cytotoxicity, and inhalation toxicity in rodents of 291 single ingredients added to cigarettes.

The studies described herein were designed to evaluate the potential influence of low-use flavoring ingredients and high-use mixed casing or flavoring ingredients on the biological activity of mainstream cigarette smoke. Test cigarettes containing flavorings or casings were analyzed and compared against an identical reference cigarette respectively produced without flavors or casings.

MATERIALS AND METHODS

Cigarette Design

In study 1, 165 low-use flavoring ingredients were added to a single test cigarette and compared to a reference cigarette without these ingredients. In study 2, eight high-use flavoring or casing ingredients were added to a single test cigarette and compared to the same reference cigarette that was used in study 1. Thus, the design covered these ingredients as well as possible interactions between them and/or their combustion or pyrolysis products. The prototype cigarettes were designed to be representative of commercial, full flavor filter cigarettes. Test and reference cigarettes were constructed with conventional commercial equipment.

The ingredients selected for evaluation in these studies comprise low-use and high-use ingredients normally utilized in the manufacture of commercial cigarettes. The point of addition was chosen to mimic actual process conditions. Study 1 and study 2 ingredients were incorporated into a flavoring or casing system at levels exceeding their normal use. Table 1 outlines the tobacco components of the blend used to construct the cigarettes in both study 1 and study 2. The blends were cased with a mixture of glycerin and water (at a ratio of 2:1) to provide the necessary moisture for standard processing. In preparation of study 1 cigarettes, the ingredients were applied at a rate of 10 kg/1000 kg leaf blend, that is, at 1% on the test cigarettes, and the casing was applied at a rate of 30 kg/1000 kg leaf blend. The study 2 ingredient system was applied at a rate of 31 kg/1000 kg leaf blend (3.1%). The 165 ingredients included in the study 1 mixture appear listed in order of descending application rate in Table 2,

TABLE 1
Blend composition of prototype cigarettes

	Percent of blend component in cigarettes	
Blend components	Tobacco wet weight	Tobacco dry weight
Burley	24	22.9
Virginia	28	25.7
Oriental	14.8	13.6
Reconstituted sheet	23.4	20.1
Expanded tobacco	9.7	8.8

along with the corresponding CAS-Number, regulatory identifiers (where applicable) and application rate. The seven casings and one flavoring included in the study 2 mixture appear listed in order of descending application rate in Table 3. Cellulose acetate filters with 32% average air dilution were used in all cigarettes. Monogram inks were not subject to these studies.

Cigarette Performance

A preliminary cigarette performance evaluation was carried out prior to the toxicology studies. Prior to characterization, the cigarettes were conditioned for a minimum of 48 h at a temperature of $22 \pm 1°C$ and a relative humidity (RH) of $60 \pm 2\%$, in accordance with ISO Standard 3402. Subsequently, the cigarettes were smoked on a 20-port Borgwaldt smoking machine under the conditions stipulated in ISO Standard 3308. Therefore, the puffing regime for mainstream smoke used a 35 ± 0.3 ml puff volume, with 2.0 ± 0.05 s puff duration once every 60 ± 0.5 s. Smoke samples were respectively collected in accordance with the analytical method.

In Vitro Study Design

The mutagenicity of total particulate matter (TPM) in study 1 and 2 cigarettes was investigated using an Ames assay protocol that conformed to OECD Guideline 471. For this purpose, prototype cigarettes containing a mixture of ingredients, reference cigarettes without these ingredients, and 2R4F cigarettes (a standard reference cigarette developed and validated by the University of Kentucky) were smoked on a Borgwaldt RM200 rotary smoking machine under the ISO standard 3308 condition. TPM was collected in a standard fiberglass (Cambridge) trap with dimethyl sulfoxide (DMSO), and the DMSO solution was stored in the dark at $-80°C$ prior to performance of the Ames assay. Each sample was tested with and without S9 metabolic activation in five strains of *Salmonella typhimurium*: TA98, TA100, TA102, TA1535, and TA1537. Evaluation of the Ames assay data was carried out in terms of the mutagenic response, taking into consideration the reproducibly dose-related increase in number of revertants, even if the increase was less than twofold. The mutagenic response to TPM from the reference and test cigarettes was compared using the linear portion of the slope (revertants/mg TPM).

TABLE 2
Ingredients added to test cigarettes in study 1

	Ingredient	CAS no.[a]	FEMA no.[b]	CFR[c]	CoE[d]	Application rate (ppm)
1	Benzyl alcohol	100-51-6	2137	172.515	58c	260
2	Immortelle extract	8023-95-8	2592	182.20	225n	156
3	Coriander oil	8008-52-4	2334	182.20	154n	65
4	Balsam peru resinoid	8007-00-9	2117	182.20	298n	65
5	Anise star oil	8007-70-3	2096	N.A.	238n	65
6	Celery seed oil	89997-35-3	2271	182.20	52n	65
7	Vanillin	121-33-5	3107	182.60	107c	65
8	Potassium sorbate	24634-61-5	2921	182.3640	N.A.	39
9	Propyl *para*-hydroxybenzoate	94-13-3	2951	172.515	N.A.	39
10	Benzoin resinoid	9000-05-9	2133	172.510	439n	26
11	Cedarwood oil	8000-27-9	N.A.	N.A.	252n	26
12	Clary extract	8016-63-5	2321	182.20	415n	26
13	Methylcyclopentenolone	80-71-7	2700	172.515	758c	26
14	Phenethyl alcohol	60-12-8	2858	172.515	68c	26
15	Piperonal	120-57-0	2911	182.60	104c	26
16	Tea extract	84650-60-2	N.A.	182.20	451n	26
17	Vanilla oleoresin	8024-06-4	3106	182.20	474n	26
18	Brandy	N.A.	N.A.	N.A.	N.A.	26
19	*trans*-Anethole	4180-23-8	2086	182.60	183c	19.5
20	Coffee extract	84650-00-0	N.A.	182.20	452n	19.5
21	5-Ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone	698-10-2	3153	N.A.	2300c	19.5
22	Propionic acid	79-09-4	2924	184.1081	3c	13
23	Acetic acid	64-19-7	2006	184.1005	2c	13
24	Amyl formate	638-49-3	2068	172.515	497c	13
25	Angelica root oil	8015-64-3	2088	182.20	56n	13
26	Beeswax absolute	8012-89-3	2126	184.1973	N.A.	13
27	Benzyl benzoate	120-51-4	2138	172.515	262c	13
28	Benzyl propionate	122-63-4	2150	172.515	413c	13
29	Cardamom oil	8000-66-6	2241	182.20	180n	13
30	beta-Carotene	7235-40-7	N.A.	184.1245	N.A.	13
31	Ethyl acetate	141-78-6	2414	182.60	191c	13
32	Ethyl butyrate	105-54-4	2427	182.60	264c	13
33	Ethyl levulinate	539-88-8	2442	172.515	373c	13
34	Eucalyptol	470-82-6	2465	172.515	182c	13
35	Geranium oil	8000-46-2	2508	182.20	324n	13
36	Labdanum resinoid	8016-26-0	2610	172.510	134n	13
37	Lavandin oil	8022-15-9	2618	182.20	257n	13
38	Maltol	118-71-8	2656	172.515	148c	13
39	Spearmint oil	8008-79-5	3032	182.20	285n	13
40	Ethyl hexanoate	123-66-0	2439	172.515	310c	10.4
41	Acetylpyrazine	22047-25-2	3126	N.A.	2286c	9.1
42	Ethylmaltol	4940-11-8	3487	172.515	692c	9.1
43	Chamomile oil, Roman	8015-92-7	2275	182.20	48n	6.5
44	Citronella oil	8000-29-1	2308	182.20	39n	6.5
45	delta-Decalactone	705-86-2	2361	172.515	621c	6.5
46	gamma-Decalactone	706-14-9	2360	172.515	2230c	6.5
47	Ethyl phenylacetate	101-97-3	2452	172.515	2156c	6.5

(Continued on next page)

TABLE 2
Ingredients added to test cigarettes in study 1 *(Continued)*

	Ingredient	CAS no.[a]	FEMA no.[b]	CFR[c]	CoE[d]	Application rate (ppm)
48	Ethyl valerate	539-82-2	2462	172.515	465c	6.5
49	Ethyl vanillin	121-32-4	2464	182.60	108c	6.5
50	Fennel sweet oil	8006-84-6	2485	182.20	200n	6.5
51	Glycyrrhizin ammoniated	53956-04-0	N.A.	184.1408	N.A.	6.5
52	gamma-Heptalactone	105-21-5	2539	172.515	2253c	6.5
53	3-Hexen-1-ol	928-96-1	2563	172.515	750c	6.5
54	3-Hexenoic acid	1577-18-0	3170	N.A.	2256c	6.5
55	Hexyl alcohol	111-27-3	2567	172.515	53c	6.5
56	Isoamyl phenylacetate	102-19-2	2081	172.515	2161c	6.5
57	Methyl phenylacetate	101-41-7	2733	172.515	2155c	6.5
58	Nerol	106-25-2	2770	172.515	2018c	6.5
59	Nerolidol	142-50-7	2272	172.515	67c	6.5
60	Peruvian (bois de rose) oil	8015-77-8	2156	182.20	44n	6.5
61	Phenylacetic acid	103-82-2	2878	172.515	672c	6.5
62	Pyruvic acid	127-17-3	2970	172.515	19c	6.5
63	Rose absolute	8007-01-0	2988	182.20	405n	6.5
64	Sandalwood oil	8006-87-9	3005	172.510	420n	6.5
65	Sclareolide	564-20-5	3794	N.A.	N.A.	6.5
66	Triethyl citrate	77-93-0	3083	184.1911	N.A.	6.5
67	2,3 5-Trimethylpyrazine	14667-55-1	3244	N.A.	735c	6.5
68	Olibanum absolute	8016-36-2	2816	172.510	93n	6.5
69	delta-Octalactone	698-76-0	3214	N.A.	2195c	6.5
70	2-Hexenal	6728-26-3	2560	172.515	748c	5.2
71	Ethyl octadecanoate	111-61-5	3490	N.A.	N.A.	5.2
72	4-Hydroxy-3-pentenoic acid lactone	591-12-8	3293	N.A.	731c	3.9
73	Methyl 2-pyrrolyl ketone	1072-83-9	3202	N.A.	N.A.	3.9
74	Methyl linoleate (48%) methyl linolenate (52%) mixture	112-63-0 301-00-8	3411	N.A.	713c	3.9
75	Petitgrain mandarin oil	8014-17-3	2854	182.20	142n	3.9
76	Propenylguaethol	94-86-0	2922	172.515	170c	3.9
77	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl) but-2-en-4-one	23696-85-7	3420	N.A.	N.A.	3.9
78	2-Propionyl pyrrole	1073-26-3	3614	N.A.	N.A.	3.9
79	Orange essence oil	8008-57-9	2825	182.20	143n	2.6
80	Benzyl phenylacetate	102-16-9	2419	172.515	232c	2.6
81	2,3-Butanedione	431-03-8	2370	184.1278	752c	1.95
82	2,3,5,6-Tetramethylpyrazine	1124-11-4	3237	N.A.	734c	1.95
83	Hexanoic acid	142-62-1	2559	172.515	9c	1.56
84	Cinnamaldehyde	104-55-2	2286	182.60	102c	1.3
85	Acetophenone	98-86-2	2009	172.515	138c	1.3
86	2-Acetylthiazole	24295-03-2	3328	N.A.	N.A.	1.3
87	Amyl alcohol	71-41-0	2056	172.515	514c	1.3
88	Amyl butyrate	540-18-1	2059	172.515	270c	1.3
89	Benzaldehyde	100-52-7	2127	182.60	101c	1.3
90	Butyl butyrate	109-21-7	2186	172.515	268c	1.3
91	Butyric acid	107-92-6	2221	182.60	5c	1.3
92	Cinnamyl alcohol	104-54-1	2294	172.515	65c	1.3

(Continued on next page)

TABLE 2
Ingredients added to test cigarettes in study 1 *(Continued)*

	Ingredient	CAS no.[a]	FEMA no.[b]	CFR[c]	CoE[d]	Application rate (ppm)
93	DL-Citronellol	106-22-9	2309	172.515	59c	1.3
94	Decanoic acid	334-48-5	2364	172.860	11c	1.3
95	para-Dimethoxybenzene	150-78-7	2386	172.515	2059c	1.3
96	3,4-Dimethyl-1,2-cyclopentanedione	13494-06-9	3268	N.A.	2234c	1.3
97	Ethylbenzoate	93-89-0	2422	172.515	261c	1.3
98	Ethyl heptanoate	106-30-9	2437	172.515	365c	1.3
99	Ethyl isovalerate	108-64-5	2463	172.515	442c	1.3
100	Ethyl myristate	124-06-1	2445	172.515	385c	1.3
101	Ethyl octanoate	106-32-1	2449	172.515	392c	1.3
102	Ethyl palmitate	628-97-7	2451	N.A.	634c	1.3
103	Ethyl propionate	105-37-3	2456	172.515	402c	1.3
104	2-Ethyl-3-methylpyrazine	15707-23-0	3155	N.A.	548c	1.3
105	Genet absolute	8023-80-1	2504	172.510	436n	1.3
106	Geraniol	106-24-1	2507	182.60	60c	1.3
107	Geranyl acetate	105-87-3	2509	182.60	201c	1.3
108	gamma-Hexalactone	695-06-7	2556	172.515	2254c	1.3
109	Hexyl acetate	142-92-7	2565	172.515	196c	1.3
110	Isoamyl acetate	123-92-2	2055	172.515	214c	1.3
111	Isoamyl butyrate	106-27-4	2060	172.515	282c	1.3
112	3,7-Dimethyl-1,6-octadiene-3-ol	78-70-6	2635	182.60	61c	1.3
113	Menthyl acetate	89-48-5	2668	172.515	206c	1.3
114	Methyl isovalerate	556-24-1	2753	172.515	457c	1.3
115	Methyl salicylate	119-36-8	2745	175.105	433c	1.3
116	3-Methylpentanoic acid	105-43-1	3437	N.A.	N.A.	1.3
117	gamma-Nonalactone	104-61-0	2781	172.515	178c	1.3
118	Oakmoss absolute	9000-50-4	2795	172.510	194n	1.3
119	Orris absolute	8002-73-1	N.A.	172.510	241n	1.3
120	Palmitic acid	57-10-3	2832	172.860	14c	1.3
121	Phenethyl phenylacetate	102-20-5	2866	172.515	234c	1.3
122	3-Propylidenephthalide	17369-59-4	2952	172.515	494c	1.3
123	Sage oil	8022-56-8	3001	182.20	61n	1.3
124	alpha-Terpineol	98-55-5	3045	172.515	62c	1.3
125	Terpinyl acetate	80-26-2	3047	172.515	205c	1.3
126	gamma-Undecalactone	104-67-6	3091	172.515	179c	1.3
127	gamma-Valerolactone	108-29-2	3103	N.A.	757c	1.3
128	3-Butylidenphthalide	551-08-6	3333	N.A.	N.A.	1.04
129	Davana oil	8016-03-3	2359	172.510	69n	0.65
130	3,5-Dimethyl-1, 2-cyclopentanedione	13494-07-0	3269	N.A.	2235c	0.65
131	Ethyl cinnamate	103-36-6	2430	172.515	323c	0.65
132	Farnesol	4602-84-0	2478	172.515	78c	0.65
133	Geranyl phenylacetate	102-22-7	2516	172.515	231c	0.65
134	alpha-Irone	79-69-6	2597	172.515	145c	0.65
135	Jasmine absolute	8022-96-6	2598	182.20	245n	0.65
136	Kola nut tincture	68916-19-8	2607	182.20	149n	0.65
137	Linalool oxide	1365-19-1	3746	172.515	N.A.	0.65
138	Linalyl acetate	115-95-7	2636	182.60	203c	0.65
139	*para*-Methoxybenzaldehyde	123-11-5	2670	172.515	103c	0.65

(Continued on next page)

TABLE 2
Ingredients added to test cigarettes in study 1 *(Continued)*

	Ingredient	CAS no.[a]	FEMA no.[b]	CFR[c]	CoE[d]	Application rate (ppm)
140	2-Methylbutyric acid	116-53-0	2695	172.515	2002c	0.65
141	Myristic acid	544-63-8	2764	172.860	16c	0.65
142	gamma-Octalactone	104-50-7	2796	172.515	2274c	0.65
143	Opoponax oil	8021-36-1	N.A.	172.510	313n	0.65
144	Tagetes oil	8016-84-0	3040	172.510	443n	0.65
145	3-Ethyl-2-hydroxy-2-cyclopenten-1-one	21835-01-8	3152	N.A.	759c	0.52
146	4-Methylacetophenone	122-00-9	2677	172.515	156c	0.26
147	Isobutyraldehyde	78-84-2	2220	172.515	92c	0.13
148	3-Methylbutyraldehyde	590-86-3	2692	172.515	94c	0.13
149	2,3-Dimethylpyrazine	5910-89-4	3271	N.A.	N.A.	0.13
150	2,5-Dimethylpyrazine	123-32-0	3272	N.A.	2210c	0.13
151	2,6-Dimethylpyrazine	108-50-9	3273	N.A.	2211c	0.13
152	Dimethyltetrahydrobenzofuranone	13341-72-5	3764	N.A.	N.A.	0.13
153	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	3658-77-3	3174	N.A.	536c	0.13
154	4-(*para*-Hydroxyphenyl)-2-butanone	5471-51-2	2588	172.515	755c	0.13
155	alpha-Ionone	127-41-3	2594	172.515	141c	0.13
156	beta-Ionone	8013-90-9	2595	172.515	142c	0.13
157	Isovaleric acid	503-74-2	3102	172.515	8c	0.13
158	Lime oil	8008-26-2	2631	182.20	141n	0.13
159	Mace absolute	8007-12-3	N.A.	182.20	296n	0.13
160	Nutmeg oil	8008-45-5	2793	182.20	296n	0.13
161	Caprylic acid	124-07-2	2799	184.1025	10c	0.13
162	Phenylacetaldehyde	122-78-1	2874	172.515	116c	0.13
163	5,6,7,8-Tetrahydroquinoxaline	34413-35-9	N.A.	N.A.	721c	0.13
164	Thyme oil	8007-46-3	3064	182.20	456n	0.13
165	Valeraldehyde	110-62-3	3098	172.515	93c	0.13

Note. "n" Follows the name of natural source of flavorings and "c" follows the number of chemical substances.
[a]Chemical Abstract Service registry number.
[b]The Flavor and Extract Manufacturers Association reference number.
[c]Code of Federal Regulations reference to Title 21 indicating regulatory status of material.
[d]Council of Europe reference number.

Inhalation Toxicity Study Design

Groups of 30 Sprague-Dawley rats of each sex were exposed by nose-only inhalation for 1 h/day, 5 days/wk for 13 consecutive weeks to concentrations of 0.06, 0.2, or 0.8 mg/L WTPM of smoke from test cigarettes containing flavoring (study 1) or to flavoring or casing ingredients (study 2). Additional groups of 30 rats/sex were exposed to the same concentrations of smoke from reference cigarettes, similar to the test cigarettes but without the flavoring or casing ingredients (as described above), or to filtered air only (sham controls). This exposure regimen (1 h/day, 5 days/wk) reflects current laboratory practices for animal inhalation studies comparing the effects of smoke from test and reference cigarettes, and does not simulate human usage patterns. However, this difference should not influence the validity of the results.

Each group of 30 rats/sex was subdivided into 2 groups: 20 rats/sex scheduled for necropsy immediately after 13 wk of exposure (interim sacrifice) and up to 10 rats/sex scheduled for necropsy following 13 wk of recovery from smoke exposure (final sacrifice). Target smoke concentrations were 0.06, 0.2, or 0.8 mg WTPM/L for the test and reference cigarettes. An additional group of 30 rats/sex served as sham controls.

Biological endpoints for the 13-wk exposure and 13-wk recovery groups included clinical appearance, body weight, organ weights, and gross and microscopic lesions. Plasma nicotine, COHb, and respiratory parameters were measured periodically during the 13-wk exposure period and clinical pathology parameters were measured at the end of the 13-wk exposure period.

Smoke Generation and Exposure System

Animal exposures were conducted in AMESA exposure units (C. H. Technologies, Westwood, NJ). The smoke exposure machines were designed to contain 30 cigarettes on a smoking head that rotated 1 revolution per minute (Baumgartner and Coggins,

TABLE 3
Ingredients added to study 2 test cigarettes

	Ingredient	CAS no.[a]	FEMA no.[b]	CFR[c]	CoE[d]	Application rate (ppm)
1	Invert sugar	8013-17-0	N.A.	184-1859	N.A.	20,000
2	Block chocolate	N.A.	N.A.	N.A.	N.A.	2,500
3	Plum extract	90082-87-4	N.A.	N.A.	371n	2,200
4	Fig extract	90028-74-3	N.A.	N.A.	198n	2,000
5	Molasse extract and tincture	68476-78-8	N.A.	N.A.	371n	2,000
6	Gentian root extract	97676-22-7	2506	172-510	214n	1,000
7	Lovage extract	8016-31-7	2650	172-510	261n	1,000
8	Peppermint oil	8006-90-4	2848	182-20	282n	250

Note. "n" Follows the name of natural source of flavorings and "c" follows the number of chemical substances.
[a]Chemical Abstract Service registry number.
[b]The Flavor and Extract Manufacturer's Association reference number.
[c]Code of Federal Regulations reference to Title 21 indicating regulatory status of material.
[d]Council of Europe reference number.

1980; Ayres et al., 1990). A vacuum port aligned with, and drew a puff from, one test or reference cigarette at a time as the head rotated. Air was drawn through the vacuum port by a peristaltic pump operating at a flow rate of ~1.05 L/min, creating a 2-s, 35-ml puff through each cigarette once each minute. The smoke vacuum flow rate was regulated by a concentration control unit consisting of a real-time aerosol monitor [(RAM)-1; MIE, Inc., Bedford, MA], a computer, and an electronic flow controller (Emerson Electric Co., Brooks Instrument Division, Hatfield, PA). The computer monitored analog voltage output of the RAM and adjusted the amount of smoke that was drawn from the glass mixing bowl by the flow controller until RAM voltage matched the calculated target voltage. The exposure units contained 3 tiers, each with 24 animal exposure ports. The exposure ports were connected to a delivery manifold, which transferred smoke to the animal breathing zone, and to an outer concentric manifold that drew the exhaled and excess smoke to an exhaust duct. Each cigarette was retained for seven puffs.

Exposure Atmosphere Characterization

The protocol-prescribed limits for the smoke concentration (WTPM/L) were target ±10% coefficient of variation (%CV). Smoke exposure concentrations were continuously monitored with a RAM at a representative exposure port. Mean exposure concentration was calculated from the mass collected on the filter and the total volume of air drawn through the filter, which was determined by the sample time and flow rate. RAM voltage readings were recorded during filter sample collection and were used to calculate a RAM response factor for subsequent exposures.

Two filters per exposure group per week were chemically analyzed for total nicotine. Nicotine standard reference material (98%) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). The WTPM:nicotine and CO:nicotine ratios were calculated for the exposure atmospheres. The concentration of CO in the test and reference atmospheres was determined using Horiba PIR-2000 CO analyzers (Horiba Instruments, Inc., Irvine, CA), monitored by DOS-based computers.

Particle size distribution of the smoke was measured using Mercer-style cascade impactors designed specifically for the size range of particles found in cigarette smoke. The mass collected on each impactor stage was analyzed gravimetrically for WTPM and the resulting data were interpreted by probit analysis (NEWCAS; Hill et al., 1977) to obtain the particle size distribution, mass median aerodynamic diameter (MMAD), and geometric standard deviation (GSD). Temperature and RH of the exposure atmospheres were measured from a representative animal exposure port once every 2 wk for each exposure group.

Animals and Animal Care

Sprague-Dawley (Crl:CD) rats 4–5 wk of age were purchased from Charles River Laboratories (Raleigh, NC), held for 13 days in quarantine status prior to initial smoke exposure. Health screens were performed following group assignment and at 24 days after arrival. These health evaluations included necropsy, microscopic examination of selected tissues and examination for parasites. The 24 days after arrival screening included serological testing for antibodies to common viral pathogens. Viral antibody testing was also performed on sera collected from 10 sentinel rats at the end of the 13-wk exposure period and from another 10 at the end of the recovery period. All sera were tested for antibodies to Sendai virus, Kilham's rat virus (KRV)/Toolan's H-1 virus, pneumonia virus of mice (PVM), rat corona virus/sialodacryoadenitis virus, and *Mycoplasma pulmonis*. During the 13-wk exposure period, the animals were housed in individual stainless-steel cages on open racks. During the recovery period, the animals were housed in individual polycarbonate cages (Lab Products, Maywood, NJ) bedded with

ALPHA-dri alpha cellulose bedding (Sheperd Specialty Papers, Kalamazoo, MI). The cage space met the requirements stated in the current *Guide for Care and Use of Laboratory Animals* (National Academy of Sciences, 1996).

Body Weight and Clinical Observations

All rats were observed twice daily for mortality and moribundity. Each rat was examined every 4 wk for clinical signs. Individual body weights were measured during the randomization procedure, on exposure day 1, biweekly thereafter, and at necropsy.

Respiratory Function Measurements

Tidal volume (TV), respiratory rate (RR), and minute volume (MV), derived from flow signals from spontaneously breathing animals, were measured in 4 rats/sex/group during wk 2, 8, and 13 using whole-body phethysmography (Coggins et al., 1981). Each animal was monitored once during a single exposure period. MV and the actual WTPM were used to estimate the average total inhaled mass for the 1-h exposure period for each animal.

Carboxyhemoglobin and Plasma Nicotine Determinations

During wk 2 and 10, blood was collected from designated animals at the end of the 1-h smoke exposure. Animals were removed from the exposure unit and bleeding was initiated within ~5 min. The blood samples were obtained from the retro-orbital plexus of carbon dioxide (CO_2)-anesthetized animals into tubes containing potassium ethylenediaminete traacetic acid (K^+-EDTA). The sample tubes were immediately placed into an ice bath and maintained under these conditions until analyzed for blood carboxyhemoglobin (COHb). Plasma nicotine was quantitatively determined using gas chromatography/mass spectrometry (GC/MS) with selected ion monitoring.

Clinical Pathology

On the day of the 13-wk interim sacrifice, the rats were anesthetized with ~70% CO_2 in room air and blood samples were obtained from the retro-orbital plexus. One sample was collected in a tube (Monoject, Sherwood Medical, St. Louis, MO) containing K^+-EDTA for hematologic determinations. Another sample was collected in a tube devoid of anticoagulant but containing a separator gel (Vacutainer, Franklin Lakes, NJ) for serum chemistry analysis. The following parameters were determined using an Abbott Cell-Dyn 3700 (Abbott Diagnostics Systems, Abbott Park, IL) multiparameter hematology instrument: white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb) concentration, volume of packed red cells (VPRC), the red cell indices (mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], and mean corpuscular hemoglobin concentration [MCHC]), platelet count, and WBC differential counts. Results of the differential cell counts were reported as both relative and absolute values. Reticulocytes were stained supravitally with new methylene blue and enumerated as reticulocytes per 1000 enthrocytes using the Miller disc method (Brecher and Schneiderman, 1950).

A Roche Hitachi 912 system (Roche Diagnostic Corp., Indianapolis, IN) chemistry analyzer was used to determine the following serum analytes: urea nitrogen (BUN), creatinine, glucose, total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), sodium, potassium, chloride, calcium, phosphorus, total bilirubin, cholesterol, and triglycerides.

Necropsy and Tissue Collection

A complete necropsy was done on all 13-wk exposure groups and 13-wk recovery group animals. Rats designated for scheduled sacrifices or sacrificed due to moribund condition were weighed and anesthetized with 70% CO_2 in air, followed by exsanguination before cessation of heartbeat. All abnormalities were recorded on the individual animal necropsy forms. Lungs, liver, kidneys, testes, adrenals, spleen, brain, and heart from all scheduled sacrifice animals were weighed. These organ weights and the body weights at necropsy were used to calculate organ:body weight ratios. In addition, organ:brain weight ratios were calculated. The time from removal of the organ until weighing was minimized to keep tissues moist.

A complete set of over 40 tissues was removed from each animal at necropsy and examined. All tissues were fixed in 10% neutral buffered formalin (NBF) except for the eyes, which were fixed in Karnovsky's fixative. After the lungs were weighed, they were perfused with 10% NBF at 25 cm hydrostatic pressure.

Histopathology

All tissues were fixed in 10% NBF for a minimum of 48 h before being trimmed. Paraffin blocks were microtomed at 5 μm. All sections were stained with hematoxylin and eosin (H&E) stains for standard histopathologic evaluation of morphologic changes. Duplicate slides of nasal tissues, larynx, lung, and trachea were stained with periodic acid-Schiff/Alcian blue (PAS/AB) stains for evaluation of goblet cell populations. The lungs, nasal cavity (four sections), nasopharynx, larynx (three cross sections), trachea (three transverse sections), tracheobronchial lymph nodes, mediastinal (thymic) lymph nodes, heart, and all gross lesions were examined microscopically. The lungs were sectioned to present a maximal section of the mainstem bronchi. The nasal cavity was prepared in four sections using the landmarks described by Young (1981). Three transverse laryngeal sections were prepared from the base of the epiglottis, the ventral pouch, and through the caudal larynx at the level of the vocal folds (Renne et al., 1992). In addition, sections of brain, adrenals, spleen, liver, kidneys, and gonads from animals in the sham control and the groups exposed to 0.8 mg/L of smoke from the test or reference cigarettes were examined microscopically. Exposure-related microscopic lesions were observed in the tissues from the rats exposed to 0.8 mg/L; target organs were examined microscopically in the lower concentration groups to ascertain a no-effect concentration.

Evaluation of Cell Proliferation Rates of Respiratory-Tract Tissues

Cell proliferation rates were measured on respiratory tract tissues collected from 10 rats of each sex from each exposure group and the sham controls necropsied immediately after 13 wk of exposure, using a monoclonal antibody to 5-bromo-2′-deoxyuridine (BrdU). Tissues evaluated using the BrdU assay included the respiratory epithelium lining the median nasal septum and distal portions of maxillary and nasal turbinates, the transitional epithelium at the base of the epiglottis, the luminal epithelium dorsolateral to the ventral pouch, the luminal epithelium lining the cranial trachea, the luminal epithelium of the mainstem bronchi and adjacent bronchioles, and selected areas of alveolar epithelium. Data from both sides of bilaterally symmetrical tissues (nose, ventral pouch, mainstem bronchi) were combined for tabulation of results.

Statistical Methods

Body weight, body weight gain, organ:body weight, and organ:brain weight ratios were statistically analyzed for each sex by exposure concentration group using the Xybion PATH/TOX system. Data homogeneity was determined by Bartlett's test. Dunnett's t-test was performed on homogeneous data to identify differences between each concentration group and the sham control group, and between corresponding concentrations of test and reference cigarette smoke-exposed groups. Nonhomogeneous data were analyzed using a modified t-test. Respiratory physiology, clinical pathology, COHb, and plasma nicotine data parameters were statistically evaluated using SAS software (Statistical Analysis System, SAS, Inc., Cary, NC). One-way analysis of variance (ANOVA) between exposure groups was first conducted, followed by Bartlett's test for homogeneity of variance. A two-sided Dunnett's multiple comparison test was employed to determine which exposure groups were different from the controls. An unpaired two-sided t-test was used to compare equivalent exposure groups between cigarette types. Differences were considered significant at $p \leq .05$. The statistical evaluation of incidence and severity of lesions was made using the Kolmogorov–Smirnov two-sample test (Siegel, 1956). All treatment group means were compared to the sham control mean, and means of groups exposed to the test cigarette smoke were compared to the corresponding reference cigarette smoke-exposed group means. Cell proliferation data were compared statistically using Tukey's studentized range test with SAS software.

RESULTS

Cigarette Performance

The results of characterization of the test and reference cigarettes for study 1 and study 2 are presented in Tables 4 and 5. These results show that the filler weight and the number of puffs per cigarette, nicotine yield, and nicotine-free dry particulate matter (NFDPM) were comparable for test and reference cigarettes in both studies. The yields of nicotine and NFDPM and the puff count were also comparable. These results are consistent with the negligible differences in the configuration of both prototype cigarettes, which basically consist of the total relative amount of flavor ingredient contained in the test cigarettes (1% or 3% of the filler weight). A comparison of the burning rates in study 1 illustrates that the addition of the ingredients had little, if any effect on the burning characteristics of the test cigarettes.

TABLE 4
Key parameters for laboratory control of prototype study 1 cigarettes

Parameter	Target	Run average: Test cigarette	Run average: Reference cigarette
Individual weights (g)			
Cigarette weight	1.012	0.963	0.965
Standard deviation	—	0.019	0.018
Non tobacco weight	0.212	0.212	0.215
Net tobacco	0.800	0.751	0.750
Air dilution (%)	32	35	34.1
Standard deviation	—	3.0	3.1
Porosity of cigarette paper (cc/min/cbar/cm^2)	50	49	49
Expanded tobacco (%)	9.7	10.1	9.1
Nicotine (mg/cig)	0.9	0.92	0.97
Nicotine (mg/puff)	n.a.	0.118	0.123
NFDPM (mg/cig)	12.0	11.3	11.5
NFDPM (mg/puff)	n.a.	1.45	1.46
CO (mg/cig)	n.a.	12.4	13.1
CO (mg/puff)	n.a.	1.59	1.66
Puffs/cigarette	n.a.	7.8	7.9
Burning rate (mg tobacco/min)	n.a.	68.1	64.4

Note. Cig, cigarette.

In Vitro Mutagenicity Assays

Figures 1, 2, 3, and 4 summarize the results of Ames assays on test cigarettes from study 1 and 2 with and without metabolic activation. TA100, TA98, and TA1537 strains showed a positive response only with metabolic activation. No response was observed in TA 102 or TA1535. No sporadic responses in revertants were recorded. The highest sensitivity and specificity of the mutagenic response were observed using TA98 with metabolic activation. From the comparison of the data obtained for the test and reference cigarettes, it was concluded that the addition of ingredients did not result in a positive mutagenic response in any of the strains under the conditions already described. Hence, the use of the tested ingredients had no influence on the mutagenic activity of the cigarettes.

TABLE 5
Key parameters for laboratory control of prototype study 2 cigarettes

Parameter	Target	Run average	
		Test cigarette	Reference cigarette
Individual weights (g)			
Cigarette weight	1.012	1.002	1.025
Standard deviation	—	0.0208	0.0173
Nontobacco weight	0.212	0.212	0.212
Net tobacco	0.800	0.790	0.813
Air dilution (%)	32	33.2	36.6
Standard deviation	—	1.6	1.4
Porosity of cigarette paper (cc/min/cbar/cm^2)	50	50	47
Expanded tobacco (%)	9.5	9.6	9.3
Nicotine (mg/cig)	0.9	0.93	0.93
Nicotine (mg/puff)	n.a.	0.112	0.107
NFDPM (mg/cig)	12.0	11.4	11.0
NFDPM (mg/puff)	n.a.	1.37	1.26
CO (mg/cig)	n.a.	12.9	12.8
CO (mg/puff)	n.a.	1.55	1.47
Puffs/cigarette	n.a.	8.3	8.7

Note. Cig, cigarette.

Exposure Atmosphere Characterization

Tables 6 and 7 summarize the exposure data for the inhalation exposure periods for study 1 and study 2. The mean exposure concentrations (WTPM) were all within 3% of the target concentration, with CVs of 6.6%, or less. Nicotine and CO concentrations correlated well with WTPM in reference and test cigarette smoke atmospheres in both study 1 and study 2. Particle sizes were slightly larger in the study 1 test and reference cigarette smokes. All concentrations of the smoke from each cigarette were highly respirable for the rat model under investigation.

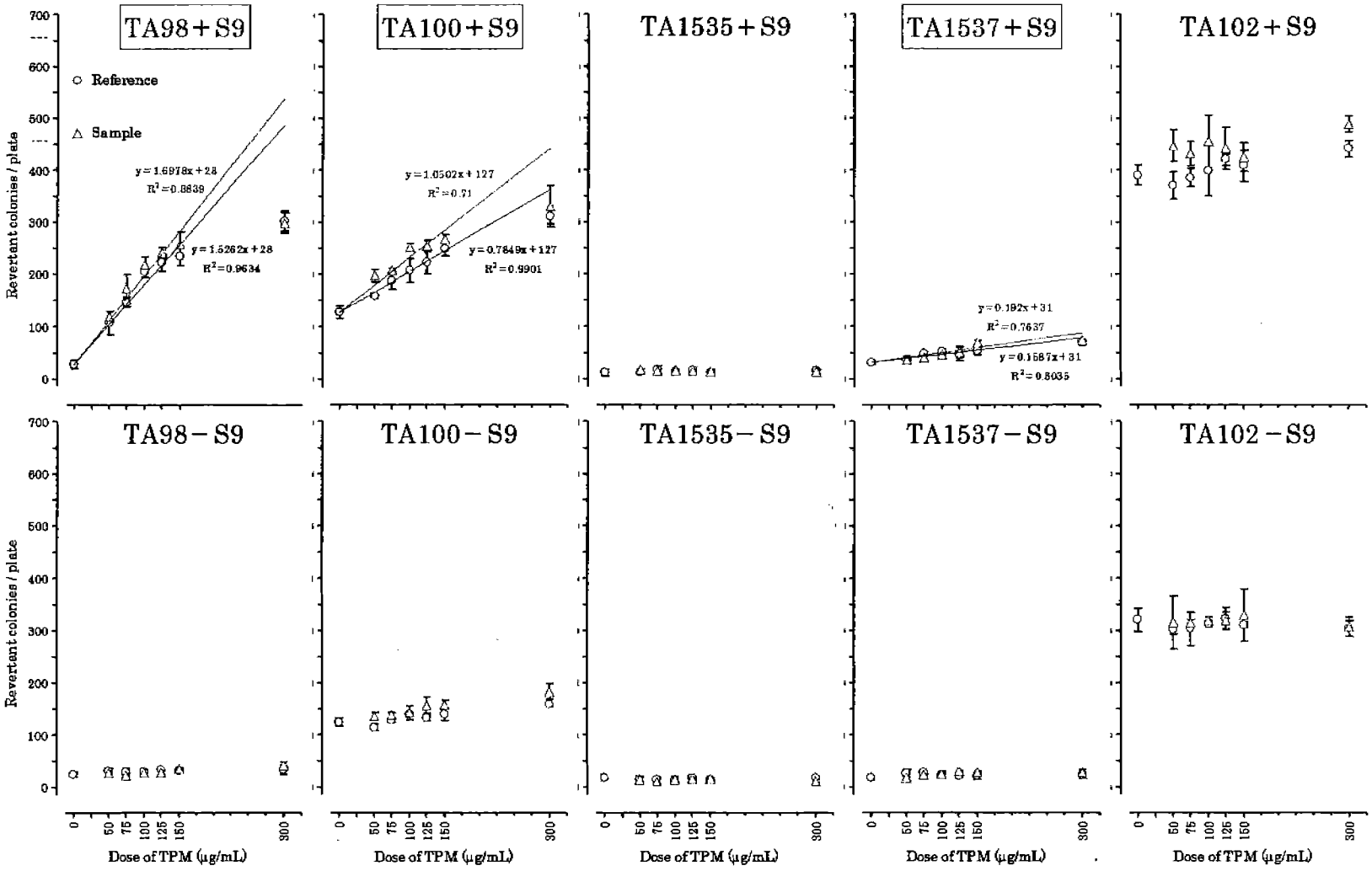
Body Weights and Clinical Observations

No significant mortality occurred in either study. Exposure-related adverse clinical signs were absent. Clinical observations noted were minor in consequence and low in incidence.

Mean body weight data for all groups on study throughout the exposure and recovery periods are illustrated in Figure 5. In study 1, mean body weights were consistently decreased compared to sham controls during the exposure period in male rats exposed to 0.8 mg/L of reference cigarette smoke and in males exposed to all 3 concentrations of test cigarette smoke. With the exception of day 71 (0.8 mg/L test), all female smoke-exposed groups in study 1 were comparable to sham control females throughout the study. In study 2, mean body weights were consistently decreased compared to sham controls in males exposed to 0.8 mg/L of test cigarette smoke and in females exposed to 0.8 mg/L of reference cigarette smoke. Mean body weights of smoke-exposed groups were similar to sham control weights during the recovery period of both study 1 and study 2. The only consistent statistical difference in body weight changes between the test and reference cigarette smoke-exposed groups in either study was the decreased mean body weight in males exposed to 0.8 mg/L of reference cigarette smoke during the exposure period of study 1.

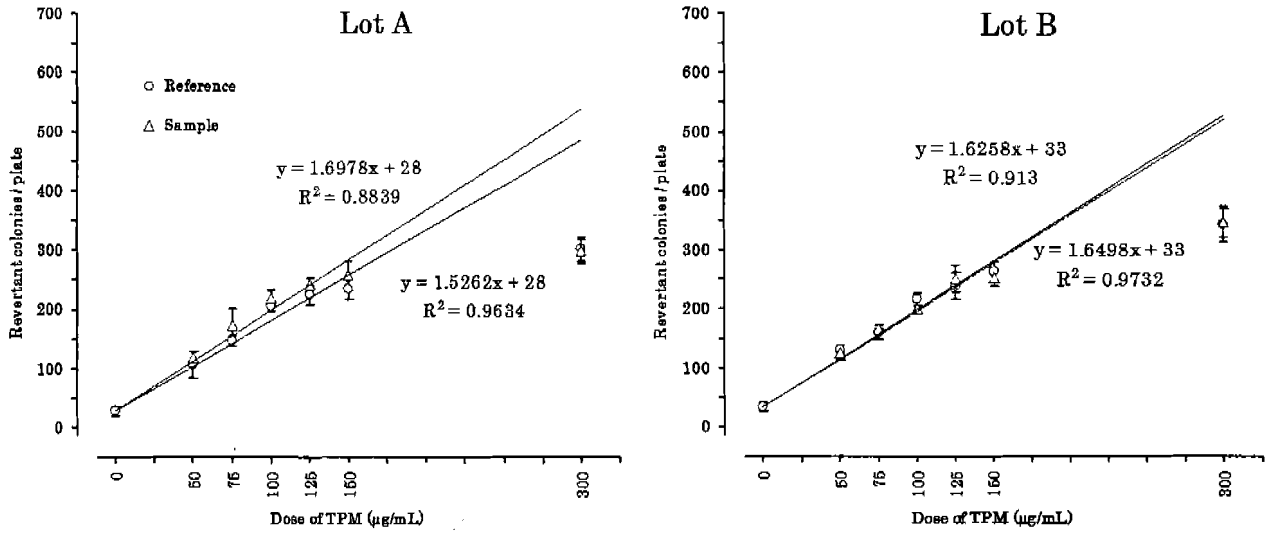
Organ Weights

Comparisons of selected group mean organ weights between smoke-exposed and sham controls in study 1 are presented in Table 8. Statistically significant differences in organ weights in groups of smoke-exposed rats were primarily low mean organ weights compared to their respective sham controls. There was no clear pattern of differences in any absolute or relative organ weight in smoke-exposed groups compared to sham controls, or in groups exposed to test versus reference cigarette smoke at either the interim sacrifice or the recovery sacrifices. Sham controls for the interim sacrifice of study 2 were inadvertently not fasted overnight prior to necropsy, which made comparison of absolute and relative organ weights of smoke-exposed and sham control groups from the interim sacrifice of questionable scientific value; thus these comparisons were not made for study 2. Statistical comparison of absolute and relative organ weights between groups exposed to test and reference cigarette smoke in study 2 showed very few statistically significant differences, none of which were considered toxicologically



N=2. Only the first lot (Lot A) is indicated in this figure.
The second lot (Lot B) showed the same tendency as the first lot.

FIG. 1. Ames assay results, study 1 cigarettes.



MEAN ± SD of Specific Activity (50 to 150 µg/plate)

Reference	1576 ± 141.9	Reference.....	1734 ± 170.9
Sample.....	1783 ± 167.3	Sample.....	1703 ± 151.2

FIG. 2. Ames assay results, study 1 with TA98 metabolic activation.

significant. Comparison of organ weights in rats necropsied following the 13-wk recovery of study 2 indicated no consistent differences between sham control and smoke-exposed groups, or between groups exposed to similar concentrations of test and reference cigarette smoke.

Respiratory Physiology

Reductions in RR and/or TV resulted in consistently lower MV in rats exposed to test or reference cigarette smoke compared to sham controls in both study 1 and study 2. There was no consistent difference in MV between groups of rats exposed to test and reference cigarette smoke in either study. Because the overall MV in study 1 was similar among groups exposed to smoke, total inhaled mass was proportional to increasing smoke concentration in this study. In study 2, decreases in MV in groups exposed to 0.8 or 0.2 mg/L compared to groups exposed to 0.06 mg/L caused total inhaled mass for the high and middle dose groups to be lower in proportion to the exposure concentration of inhaled smoke.

Clinical Pathology

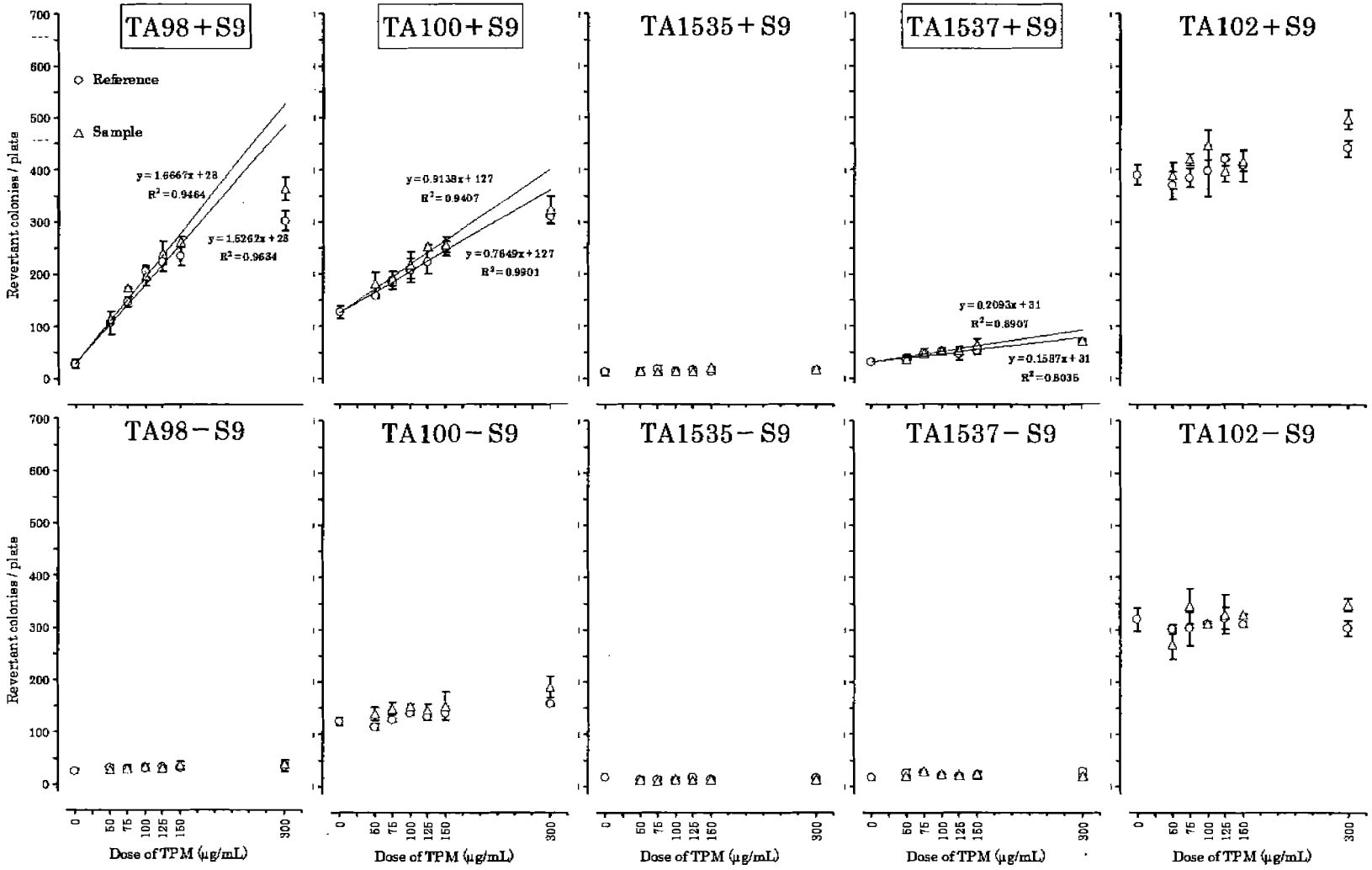
There were occasional statistically significant differences in hematology and clinical chemistry parameters from control values in groups exposed to smoke from test or reference cigarettes in both study 1 and study 2. These differences did not occur in a dose-response pattern and were well within ±2 standard deviations of historic values for control Sprague-Dawley rats of comparable age. There were also statistically significant differences in several hematology and clinical chemistry parameters between groups exposed to similar concentrations of test and reference cigarette smoke. These differences are not considered to be of toxicologic significance, nor were they exposure related.

Whole-blood COHb levels were increased in a graded dose-response fashion as a function of exposure concentration for all test and reference cigarette smoke-exposed groups in both studies. In study 2 rats bled during exposure wk 2, there was a statistically significant decrease in COHb levels in both sexes exposed to 0.8 mg/L of test cigarette smoke and in females exposed to 0.2 mg/L of test cigarette smoke, compared to groups exposed to reference cigarette smoke. There were no other clear differences in whole blood COHb levels between the test and reference cigarette groups at equivalent exposure levels in either study.

Plasma nicotine levels increased in a graded dose-response fashion for test and reference males and female groups in both studies. In study 2, test female groups exposed to 0.8 mg/L had significantly lower plasma nicotine levels than the 0.8 mg/L reference females at both 2- and 10-wk sampling. Comparing males to females at all exposure levels for test and reference cigarettes, the females consistently had higher plasma nicotine levels in both studies.

Pathology

Few gross lesions were observed in either study, with no evidence of changes attributable to exposure to smoke from the test



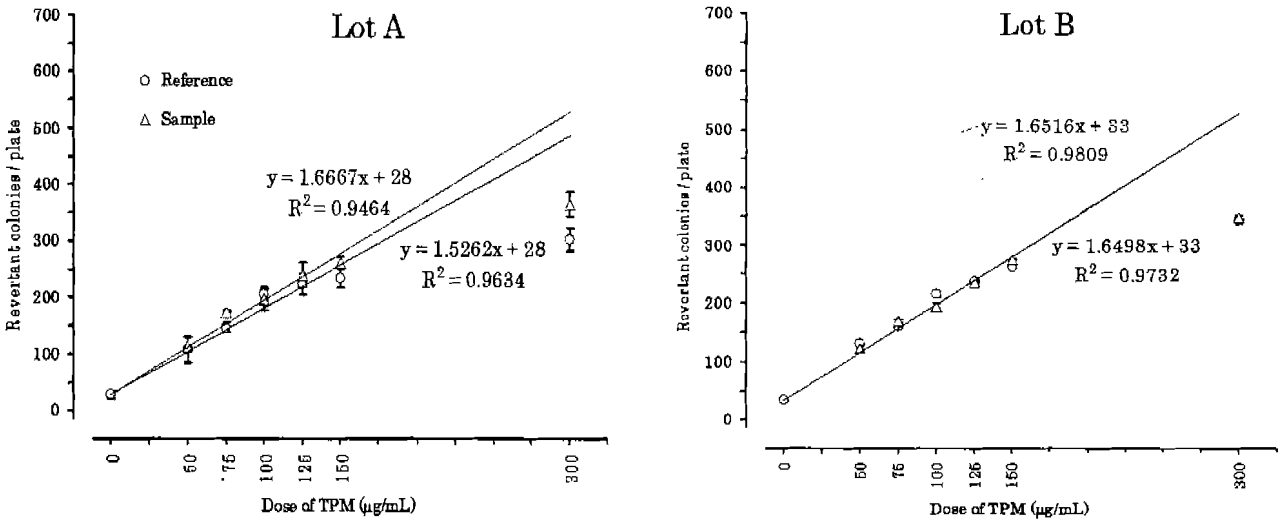
N=2. Only the first lot (Lot A) is indicated in this figure.
The second lot (Lot B) showed the same tendency as the first lot .

FIG. 3. Ames assay results, study 2 cigarettes.

TABLE 6
Study 1, exposure concentration data for rats exposed to mainstream smoke from test or reference cigarettes

	Concentration [mean ± SD (%CV)]				
	Measured exposure concentration (mg WTPM/L; $n = 126$)	Nicotine concentration (μg/L; $n = 28$)	CO concentration (ppm; $n = 63$)	Percent of target WTPM concentration (mean ± SD)	Particle size (MMAD, μm)
Test target exposure concentration (mg WTPM/L)					
0.800	0.787 ± 0.035 (4.4)	68.2 ± 2.5 (3.7)	584 ± 27 (4.6)	98.4 ± 4.3	0.73 ± 0.08
0.200	0.199 ± 0.009 (4.5)	15.5 ± 1.0 (6.5)	144 ± 6 (4.2)	99.3 ± 4.3	0.74 ± 0.12
0.060	0.061 ± 0.004 (6.6)	4.4 ± 0.5 (11.4)	47 ± 3 (6.4)	101 ± 6	0.69 ± 0.09
Reference target exposure concentration (mg WTPM/L)					
0.800	0.795 ± 0.023 (2.9)	70.1 ± 2.1 (2.9)	608 ± 20 (3.3)	99.4 ± 2.7	0.74 ± 0.08
0.200	0.202 ± 0.004 (2.0)	15.8 ± 0.7 (4.5)	147 ± 4 (2.7)	101 ± 2	0.72 ± 0.07
0.060	0.060 ± 0.002 (3.3)	4.4 ± 0.4 (9.8)	50 ± 2 (4.8)	100 ± 4	0.74 ± 0.10

Note. CO, carbon monoxide; WTPM, wet total particulate matter.



MEAN±SD of Specific Activity (50 to 150 µg/plate)

Reference.....	1576±141.9	Reference.....	1734±170.9
Sample.....	1726±138.6	Sample-1.....	1701±107.9

FIG. 4. Ames assay results, study 2 cigarettes with TA98 metabolic activation.

TABLE 7
Study 2, exposure concentration data for rats exposed to smoke from test or reference cigarettes

	Concentration [mean ± SD (%CV)]				
	Measured exposure concentration (mg WTPM/L; $n = 134$)	Nicotine concentration (μg/L; $n = 28$)	CO concentration (ppm; $n = 67$)	Percent of target WTPM concentration (mean ± SD)	Particle size (MMAD, μm)
Test target exposure concentration (mg WTPM/L)					
0.8	0.798 ± 0.040 (5.0)	56.8 ± 2.6 (4.6)	646 ± 34 (5.3)	100 ± 5	0.65 ± 0.01
0.2	0.194 ± 0.007 (3.6)	12.9 ± 0.6 (4.7)	158 ± 9 (5.7)	97 ± 4	0.62 ± 0.04
0.060	0.060 ± 0.002 (3.3)	4.0 ± 0.2 (5.0)	54 ± 3 (5.6)	100 ± 3	0.66 ± 0.03
Reference target exposure concentration (mg WTPM/L)					
0.8	0.784 ± 0.031 (4.0)	55.1 ± 2.3 (4.2)	676 ± 31 (4.6)	98 ± 4	0.57 ± 0.03
0.2	0.201 ± 0.004 (1.8)	13.0 ± 0.4 (3.4)	170 ± 15 (8.7)	100 ± 2	0.64 ± 0.07
0.060	0.060 ± 0.002 (3.3)	4.1 ± 0.2 (4.4)	57 ± 3 (5.8)	99 ± 3	0.66 ± 0.06

Note. CO, carbon monoxide; WTPM, wet total particulate matter.

or the reference cigarettes. Exposure to smoke from reference or test cigarettes in both studies induced concentration-related proliferative, metaplastic, and inflammatory microscopic lesions in the respiratory tract after 13 wk of exposure. The incidence of exposure-related respiratory-tract lesions observed at microscopic examination of tissues from rats necropsied at the interim sacrifice immediately following 13 wk of exposure is summarized in Table 9 for study 1 and Table 10 for study 2.

Hyperplasia of respiratory epithelium lining the anterior nasal cavity was present in all rats exposed to 0.8 mg/L in both studies, a few rats exposed to 0.2 mg/L in both studies, and in 3/40 rats exposed to 0.06 mg/L in study 1. Areas most severely and most frequently affected were the distal portions of the nasal and maxillary turbinates in sections of nose just caudal to the incisor teeth. In affected rats, the epithelium in the distal turbinates was up to six cells thick. There was also a clear dose response in the severity of nasal respiratory epithelial hyperplasia, with severity ranging from minimal to moderate. Comparison of incidence and severity data for nasal respiratory epithelial hyperplasia in rats exposed to similar concentrations of smoke from the test and reference cigarettes did not indicate any statistically significant differences in either study. Minimal goblet-cell hyperplasia was observed in the mucosal epithelium lining the median nasal septum in some smoke-exposed and sham control rats. Although not statistically significant compared to concurrent sham controls, the incidence of nasal goblet cell hyperplasia in male rats exposed to the 0.8-mg/L concentration of smoke from the reference cigarette or test cigarette in study 1 were considered to be toxicologically significant. There was no clear difference in the incidence of goblet cell hyperplasia between groups exposed to similar concentrations of reference and test cigarette smoke in either study.

Exposure to smoke from the reference or test cigarette in both study 1 and study 2 induced squamous metaplasia, hyperplasia, and hyperkeratosis of the transitional epithelium lining the base of the epiglottis and the epithelium lining the dorsal border of the ventral pouch and the adjacent laryngeal lumen. In control rats, the epithelium lining the base of the epiglottis was a mixture of ciliated columnar epithelium and slightly flattened, oval, rounded, or cuboidal cells one or two cells thick over a poorly defined basal cell layer (Renne et al., 1992). In affected smoke-exposed rats, the base of the epiglottis was covered by a stratified squamous epithelium up to eight cells thick with a variably keratinized surface layer and a distinct basal cell layer. There was a concentration-related increase in severity of squamous metaplasia and hyperplasia of epiglottis epithelium in rats exposed to test or reference cigarette smoke. Statistical analysis did not indicate any significant differences in incidence or severity of these lesions between test and reference cigarette smoke-exposed groups in either study. Hyperkeratosis (accumulation of keratinized squamous cells on the surface) was observed in association with squamous metaplasia of the epithelium lining the base of the epiglottis in most rats exposed to smoke from reference or test cigarettes. Comparison of incidence/severity of hyperkeratosis in the epiglottis between test and reference cigarette smoke-exposed groups indicated a statistically

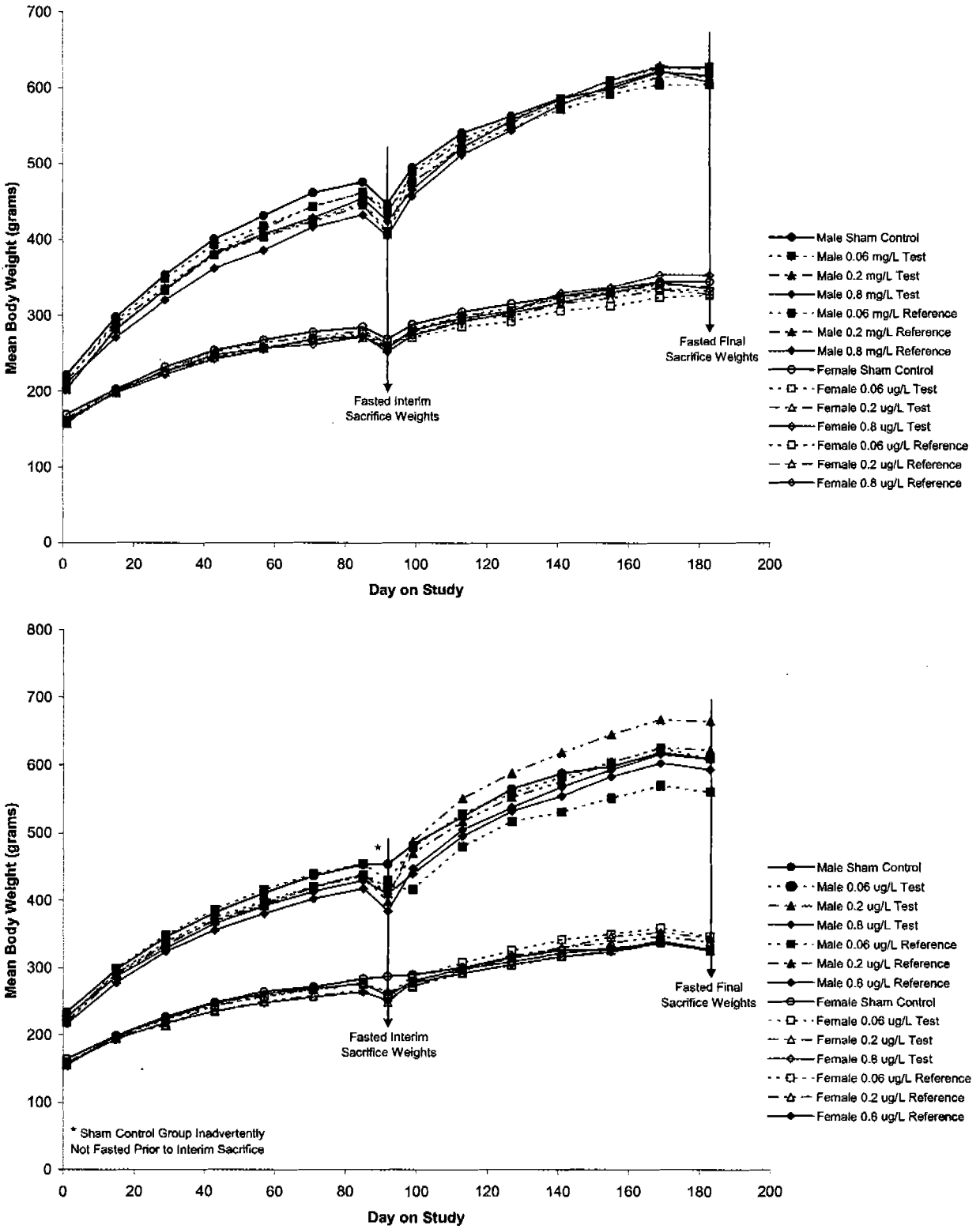


FIG. 5. Body weights, study 1 (top) and study 2 (bottom).

TABLE 8
Organ weights for rats exposed to smoke from study 1 cigarettes ($n = 20$, g ± SD)

		Test			Reference		
	Sham control	0.06 mg WTPM/L	0.2 mg WTPM/L	0.8 mg WTPM/L	0.06 mg WTPM/L	0.2 mg WTPM/L	0.8 mg WTPM/L
Males							
Heart	1.60 ± 0.16	1.48 ± 0.15[a,b]	1.43 ± 0.16[a,c]	1.55 ± 0.15	1.60 ± 0.13	1.57 ± 0.16	1.52 ± 0.15
Kidneys	3.39 ± 0.33	3.17 ± 0.39	2.92 ± 0.30[a,c]	3.05 ± 0.33[a]	3.38 ± 0.33	3.20 ± 0.31	3.02 ± 0.27[a]
Lungs	1.95 ± 0.22	1.89 ± 0.17	1.82 ± 0.23[c]	1.93 ± 0.14	2.02 ± 0.28	1.98 ± 0.26	1.89 ± 0.15
Adrenals	0.066 ± 0.010	0.066 ± 0.012	0.059 ± 0.010	0.064 ± 0.012	0.062 ± 0.007	0.064 ± 0.008	0.063 ± 0.008
Females							
Heart	1.06 ± 0.09	1.02 ± 0.10	1.00 ± 0.10[c]	1.05 ± 0.12	1.03 ± 0.09	1.07 ± 0.09	1.09 ± 0.12
Kidneys	2.18 ± 0.21	2.02 ± 0.24	1.90 ± 0.19[a]	1.93 ± 0.18[a]	2.04 ± 0.21	1.99 ± 0.19[a]	1.95 ± 0.19[a]
Lungs	153 ± 0.13	1.50 ± 0.13	1.52 ± 0.17[c]	1.52 ± 0.15	1.55 ± 0.14	1.50 ± 0.17	1.60 ± 0.19
Adrenals	0.080 ± 0.010	0.081 ± 0.011	0.078 ± 0.008	0.082 ± 0.012	0.078 ± 0.008	0.080 ± 0.010	0.081 ± 0.013

[a] $p < .05$, Dunnett's t-test of significance, compared to sham control.
[b] $p < .05$, Dunnett's t-test of significance, compared to 0.06 reference group.
[c] $p < .05$, Dunnett's t-test of significance, compared to 0.2 reference group.

significant difference only in the 0.06-mg/L groups from study 1, in which females exposed to test cigarette smoke had a higher incidence/severity than females exposed to reference cigarette smoke. Chronic inflammation was present in the submucosa of the epiglottis in some rats exposed to reference or test cigarette smoke in study 1, most frequently in rats exposed to the 0.8 mg/L smoke concentration. Squamous metaplasia, hyperplasia, and hyperkeratosis were also present in the epithelium lining the opening of the ventral pouch and the adjacent laryngeal lumen in most rats exposed to smoke from the test or reference cigarette in both studies. In control rats, the epithelium lining the opening of the ventral pouch and adjacent laryngeal lumen was slightly flattened, oval, rounded, or cuboidal cells one or two cells thick with no discernible basal cell layer (Renne et al., 1992). In affected smoke-exposed rats, this area was covered by a stratified squamous epithelium from three to six cells thick with a variably keratinized surface layer and a distinct basal cell layer. Comparison of incidence/severity of lesions at this site between test and reference cigarette smoke-exposed groups did not indicate any statistically significant differences in either study. Minimal or mild squamous metaplasia of the mucosal epithelium lining the caudal larynx was observed in 2/20 rats exposed to the 0.8 mg/L concentration of smoke from the test cigarette and 1/20 rats exposed to the 0.8 mg/L concentration of smoke from the reference cigarette in study 1.

Exposure to smoke from reference or test cigarettes induced a dose-related increase in minimal hyperplasia of the mucosal epithelium lining the tracheal lumen in both sexes of rats in study 1 and in males in study 2. Comparison of incidence in groups exposed to similar concentrations of smoke from test and reference cigarettes did not indicate any statistical differences in either study.

There were increased numbers of macrophages diffusely scattered through the pulmonary alveoli of rats exposed to smoke from reference or test cigarettes in both studies, compared to concurrent controls. There was some evidence of a dose response in the incidence and severity of macrophage accumulation in alveoli of smoke-exposed rats. This increase was graded as minimal in the vast majority of affected rats. Comparison of incidence and severity data for macrophages in alveoli of rats exposed to smoke from the test and reference cigarettes did not indicate any statistically significant differences. Minimal goblet-cell hyperplasia was observed in AB/PAS-stained sections of the mainstem bronchi of some rats exposed to smoke from reference or test cigarettes in both studies. There was some evidence of a dose response in the incidence of this lesion. Analysis of data indicated a statistically significant increase compared to controls in rats of both sexes exposed to the 0.8 mg/L concentration of smoke from reference cigarettes and in female rats exposed to the 0.8-mg/L concentration of smoke from the test cigarette in study 1, and in both sexes exposed to 0.8 mg/L of reference cigarette smoke in study 2. The incidence (7/20) of goblet-cell hyperplasia in males exposed to the 0.8-mg/L concentration of smoke from the test cigarette in both studies, although not statistically significant, was considered to be toxicologically significant. The incidence of bronchial goblet-cell hyperplasia was slightly higher in male rats exposed to smoke from reference cigarettes compared to similar concentrations of smoke from test cigarettes, but comparison of incidence in groups exposed to similar concentrations of smoke from test and reference cigarettes did not indicate any statistical differences. There was a very low incidence of a variety of microscopic lesions in other tissues examined in both studies, with no evidence of an effect of exposure to smoke from the reference or test cigarette on these tissues.

TABLE 9
Study 1, summary of microscopic observations with average severity in rats

Organ/diagnosis	Incidence of lesions (mean severity, if applicable) by target exposure concentration (mg WTPM/L)						
		Test			Reference		
	Sham controls	0.06	0.2	0.8	0.06	0.2	0.8
				Males			
Nose/turbinates	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Respiratory epithelium, hyperplasia	0[b] (0.0)	2 (0.2)	4 (0.3)	20 (2.2)	1 (0.1)	8 (0.4)	20 (2.1)
Goblet-cell hyperplasia	2 (0.1)	6 (0.3)	3 (0.2)	9 (0.5)	5 (0.3)	5 (0.3)	10 (0.5)
Suppurative inflammation	2 (0.2)	2 (0.3)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	1 (0.1)
Larynx	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Epiglottis, squamous metaplasia	0 (0.0)	20 (2.2)	20 (2.9)	20 (3.0)	20 (2.1)	20 (2.9)	20 (3.1)
Epiglottis, epithelial hyperplasia	0 (0.0)	20 (2.2)	20 (2.9)	20 (3.0)	20 (2.1)	20 (2.9)	20 (3.0)
Epiglottis, hyperkeratosis	0 (0.0)	9 (0.5)	20 (1.4)	19 (1.9)	16 (0.9)	20 (1.8)	20 (1.9)
Ventral pouch, squamous metaplasia	0 (0.0)	12 (0.7)	20 (2.4)	20 (2.8)	7 (0.5)	19 (2.7)	20 (2.9)
Ventral pouch, epithelial hyperplasia	0 (0.0)	12 (0.7)	20 (2.4)	20 (2.8)	7 (0.5)	19 (2.7)	20 (2.9)
Ventral pouch, hyperkeratosis	0 (0.0)	0 (0.0)	9 (0.6)	19 (1.4)	1 (0.2)	17 (1.4)	18 (1.5)
Chronic inflammation	0 (0.0)	2 (0.1)	8 (0.4)	16 (0.9)	0 (0.0)	4 (0.2)	13 (0.7)
Caudal larynx, squamous metaplasia	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	0 (0.0)
Trachea	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Epithelial hyperplasia	1 (0.1)	6 (0.3)	6 (0.3)	18 (0.9)	5 (0.3)	12 (0.6)	16 (0.8)
Lung	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Alveoli, macrophages	3 (0.2)	15 (0.8)	14 (0.7)	20 (1.4)	8 (0.4)	11 (0.6)	20 (1.1)
Bronchi, goblet-cell hyperplasia	0 (0.0)	1 (0.1)	1 (0.1)	7 (0.4)	3 (0.2)	4 (0.2)	11 (0.6)
Alveoli, hemorrhage	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)
				Females			
Nose/turbinates	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Respiratory epithelium, hyperplasia	0[b] (0.0)	0 (0.0)	7 (0.4)	20 (2.0)	0 (0.0)	3 (0.2)	20 (2.1)
Goblet-cell hyperplasia	2 (0.1)	2 (0.1)	2 (0.1)	7 (0.4)	2 (0.1)	2 (0.1)	4 (0.2)
Suppurative inflammation	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Larynx	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Epiglottis, squamous metaplasia	0 (0.0)	20 (2.2)	20 (3.0)	20 (3.1)	20 (2.2)	20 (2.6)	20 (3.1)
Epiglottis, epithelial hyperplasia	0 (0.0)	20 (2.2)	20 (3.0)	20 (3.1)	20 (2.2)	20 (2.6)	20 (3.0)
Epiglottis, hyperkeratosis	0 (0.0)	19 (1.4)[c]	20 (2.2)	20 (2.2)	13 (0.7)	20 (2.0)	20 (2.1)
Ventral pouch, squamous metaplasia	0 (0.0)	10 (0.6)	20 (2.7)	20 (3.0)	12 (0.8)	20 (2.7)	20 (2.9)
Ventral pouch, epithelial hyperplasia	0 (0.0)	10 (0.6)	20 (2.7)	20 (3.0)	12 (0.8)	20 (2.7)	20 (2.9)
Ventral pouch, hyperkeratosis	0 (0.0)	0 (0.0)	15 (1.3)	20 (1.8)	1 (0.1)	18 (1.5)	18 (1.5)
Chronic inflammation	0 (0.0)	3 (0.2)	2 (0.2)	10 (0.6)	0 (0.0)	4 (0.2)	17 (1.0)
Caudal larynx, squamous metaplasia	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	1 (0.1)
Trachea	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Epithelial hyperplasia	1 (0.1)	2 (0.1)	8 (0.4)	12 (0.6)	3 (0.2)	7 (0.4)	18 (0.9)
Lung	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Alveoli, macrophages	3 (0.2)	10 (0.5)	13 (0.7)	20 (1.2)	12 (0.6)	17 (0.9)	20 (1.3)
Bronchi, goblet-cell hyperplasia	0 (0.0)	2 (0.1)	3 (0.2)	10 (0.5)	1 (0.1)	4 (0.2)	13 (0.7)
Alveoli, hemorrhage	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Note. Severity: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.
[a]Number of tissues or animals examined.
[b]Number of diagnoses made.
[c]$p < .05$, Kolmogorov–Smirnov test, compared to 0.06-mg/L reference group.

TABLE 10
Study 2, summary of microscopic observations with average severity in rats

Organ/diagnosis	Incidence of lesions (mean severity, if applicable) by target exposure concentration (mg WTPM/L)						
		Test			Reference		
	Sham controls	0.06	0.2	0.8	0.06	0.2	0.8
				Males			
Nose/turbinates	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Respiratory epithelium, hyperplasia	0[b] (0.0)	0 (0.0)	2 (0.1)	20 (2.0)	0 (0.0)	4 (0.2)	20 (1.9)
Goblet-cell hyperplasia	2 (0.1)	3 (0.2)	3 (0.2)	3 (0.2)	3 (0.2)	4 (0.2)	3 (0.2)
Suppurative inflammation	0 (0.0)	2 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)
Larynx	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Epiglottis, squamous metaplasia	0 (0.0)	20 (1.8)	20 (2.4)	20 (3.0)	20 (1.9)	20 (2.5)	20 (3.0)
Epiglottis, epithelial hyperplasia	0 (0.0)	20 (1.8)	20 (2.4)	20 (3.0)	20 (1.9)	20 (2.5)	20 (3.0)
Epiglottis, hyperkeratosis	0 (0.0)	6 (0.4)	15 (1.2)	20 (2.0)	13 (1.0)	20 (1.8)	20 (2.1)
Ventral pouch, squamous metaplasia	0 (0.0)	1 (0.1)	18 (1.4)	20 (1.8)	1 (0.1)	16 (1.2)	20 (1.8)
Ventral pouch, epithelial hyperplasia	0 (0.0)	1 (0.1)	18 (1.4)	20 (1.8)	1 (0.1)	16 (1.2)	20 (1.8)
Ventral pouch, hyperkeratosis	0 (0.0)	0 (0.0)	6 (0.4)	16 (1.2)	0 (0.0)	5 (0.4)	16 (1.0)
Trachea	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Epithelial hyperplasia	2 (0.1)	8 (0.4)	9 (0.5)	11 (0.6)	6 (0.3)	8 (0.4)	10 (0.5)
Lung	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Alveoli, macrophages	4 (0.2)	11 (0.6)	16 (0.9)	20 (1.4)	11 (0.6)	14 (0.7)	20 (1.4)
Alveoli, hemorrhage	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	0 (0.0)
Chronic inflammation	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Bronchi, goblet-cell hyperplasia	0 (0.0)	1 (0.1)	1 (0.1)	4 (0.2)	0 (0.0)	1 (0.1)	9 (0.5)
				Females			
Nose/turbinates	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Respiratory epithelium, hyperplasia	0[b] (0.0)	0 (0.0)	4 (0.2)	20 (1.5)	0 (0.0)	4 (0.2)	20 (1.6)
Goblet-cell hyperplasia	3 (0.2)	3 (0.2)	5 (0.3)	5 (0.3)	5 (0.3)	2 (0.1)	8 (0.4)
Suppurative inflammation	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)
Larynx	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Epiglottis, squamous metaplasia	0 (0.0)	20 (1.9)	20 (2.8)	20 (2.8)	20 (1.8)	20 (2.6)	20 (2.6)
Epiglottis, epithelial hyperplasia	0 (0.0)	20 (1.9)	20 (2.8)	20 (2.8)	20 (1.8)	20 (2.6)	20 (2.6)
Epiglottis, hyperkeratosis	0 (0.0)	16 (1.0)	20 (2.0)	20 (2.2)	15 (0.9)	20 (1.6)	20 (2.4)
Ventral pouch, squamous metaplasia	0 (0.0)	1 (0.1)	15 (1.2)	19 (1.9)	2 (0.1)	16 (1.1)	20 (2.0)
Ventral pouch, epithelial hyperplasia	0 (0.0)	1 (0.1)	14 (1.1)	19 (1.9)	2 (0.1)	16 (1.1)	20 (2.0)
Ventral pouch, hyperkeratosis	0 (0.0)	0 (0.0)	6 (0.5)	18 (1.4)	0 (0.0)	9 (0.6)	20 (1.7)
Trachea	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Epithelial hyperplasia	1 (0.1)	0 (0.0)	1 (0.1)	2 (0.1)	2 (0.1)	1 (0.1)	2 (0.1)
Lung	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]	20[a]
Alveoli, macrophages	3 (0.2)	9 (0.5)	10 (0.5)	19 (1.1)	10 (0.5)	10 (0.5)	17 (1.0)
Perivascular lymphoid infiltrate	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	0 (0.0)
Alveoli, hemorrhage	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Chronic inflammation	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Bronchi, goblet-cell hyperplasia	0 (0.0)	1 (0.1)	0 (0.0)	7 (0.4)	3 (0.2)	4 (0.2)	10 (0.5)

Note. Severity: 1 = minimal; 2 = mild; 3 = moderate; 4 = marked.
[a]Number of tissues or animals examined.
[b]Number of diagnoses made.

Examination of tissue sections from rats necropsied at the end of the recovery period demonstrated nearly complete regression of nasal and tracheal lesions and a substantial decrease in the incidence and severity of smoke-induced lesions in the larynx and lungs in rats exposed to smoke from test or reference cigarettes in both studies. Macrophages observed in alveoli of smoke-exposed and control recovery group rats were in small focal aggregates, as opposed to the diffuse distribution of macrophages in lungs of rats necropsied at the interim sacrifice. There was no statistically significant difference in the incidence or severity of respiratory-tract lesions between recovery group rats previously exposed to similar concentrations of test and reference cigarette smoke in either study.

Evaluation of Cell Proliferation Rates

There was a dose-related trend toward higher mean nuclear labeling rates in the epithelium lining the median nasal septum in groups exposed to progressively higher concentrations of test or reference cigarette smoke compared to sham controls, but the increases were statistically significant only in females exposed to 0.8 mg/L of test cigarette smoke in study 1 and males exposed to 0.8 mg/L of reference cigarette smoke in study 2. Mean nuclear labeling rates of nasal epithelium lining the distal portions of the nasal and maxillary turbinates were statistically increased compared to control rates in both sexes of rats exposed to 0.8 mg/L of smoke from the test or reference cigarettes in both studies. Mean labeling rates in nasal and maxillary turbinates of study 1 males exposed to 0.8 mg/L of test cigarette smoke were statistically increased compared to labeling rates at these sites in males exposed to the same concentration of reference cigarette smoke.

Mean nuclear labeling rates in laryngeal epithelium were increased compared to sham control groups at all dose levels in both studies. Labeling rates in laryngeal epithelium were statistically different between several test and reference cigarette smoke-exposed groups in both studies, with no clear trend. The histopathology findings of laryngeal epithelial hyperplasia in smoke-exposed rats confirmed the relative sensitivity of these laryngeal sites to smoke-induced hyperplastic changes.

Mean nuclear labeling rates in the tracheal epithelium of rats exposed to smoke from test or reference cigarettes were not clearly different from those of sham controls of the same sex in either study. Labeling rates of bronchial, bronchiolar, and alveolar epithelium in both studies were difficult to evaluate due to wide standard deviations, low labeling rates, and variable sample sizes, and therefore labeling data from these sites were not used in evaluating effects of smoke exposure.

DISCUSSION

The studies described here were designed to evaluate the potential influence of ingredients on the chemical composition and the biological activity of mainstream cigarette smoke. Test cigarettes containing flavorings or casings were analyzed and compared against reference cigarettes identical except produced without flavors or casings. The configuration and ISO-condition tar, nicotine, and CO yields of all cigarettes investigated are representative of American blend cigarettes. Both test and reference cigarettes had the same tobacco blend and humectant composition (glycerine plus water) and were prepared by the same manufacturing process. Similarly, identical nontobacco materials (NTM) were used throughout. The weight of the filler remained constant between test and reference cigarettes. These studies illustrate that the application of 165 low-use flavoring or 8 high-use flavoring or casing ingredients had little, if any, observable effect on the deliveries or physical parameters of the cigarettes.

From comparison of the mutagenicity data obtained in Ames assays of studies 1 and 2 test and reference cigarettes, it was concluded that the addition of these ingredients did not increase the mutagenic response of any of the strains of *Salmonella typhimurium* under the conditions described, and the results did not suggest any mutagenic activity of the added ingredients.

The objectives of the two inhalation toxicity studies were to compare the biologic activity of mainstream smoke from the two test cigarettes with reference cigarettes in a series of two 13-wk inhalation exposures, each followed by a 13-wk recovery period. Data collected during the 13-wk exposures confirmed that both the particulate (WTPM, nicotine) and vapor (CO) phases of the inhalation atmospheres presented to the rats were well controlled and provided appropriate data for comparison of the responses of the study animals to smoke from the two cigarettes under investigation in each of the two studies. WTPM was used as the basis for exposure concentration in these studies, since the predominant known toxicologic effects of cigarette smoke are associated with the mainstream particulate phase (Coggins et al., 1980).

Blood COHb concentrations demonstrated that exposure of rats to smoke from either the test or reference cigarette resulted in reproducible biomarkers of exposure consistent with the concentration of CO in the smoke. Samples taken for plasma nicotine analysis confirmed exposure to nicotine in test or reference smoke, which resulted in exposure-related increases in plasma nicotine concentrations.

The only occurrence during either study that affected the utility of the data was the failure to fast the sham control rats prior to necropsy at the interim sacrifice immediately following the exposure period in study 2. This error did not allow direct comparison of the body and organ weights of controls with smoke-exposed groups sacrificed at that time point.

Other investigations have noted effects similar to those we observed of cigarette smoke exposure on body weight, including the relative resistance of females to this change (Coggins et al., 1989; Baker et al., 2004). We concluded that the decreased body weights in smoke-exposed groups in both studies compared to sham controls were the result of smoke exposure. However, we do not consider these effects on body weight to be toxicologically significant due to their recovery after smoke exposure was terminated, and due to the lack of any concurrent clinical observations that would indicate any significant dysfunction.

In study 1 there were a number of statistically significant differences in absolute or relative organ weights between test or reference cigarette smoke-exposed groups and sham controls necropsied immediately following 13 wk of smoke exposure. However, these statistical differences showed no clear dose-response pattern, and no exposure-related histopathologic effects were observed in any weighed organ except the lungs. It is possible that the increased lung/body weight ratios in study 1 rats exposed to 0.8-mg/L of smoke from test or reference cigarettes were related to the minimal increase in numbers of macrophages in alveoli of these rats. These increases in lung/body weight ratio more likely reflect the decreased body weight in these groups at the interim sacrifice. In any case, these and the other statistical differences in absolute or relative organ weights in smoke-exposed rats compared to sham controls are not considered toxicologically significant. There was no consistent difference in organ weights between groups of rats exposed to similar concentrations of test and reference cigarette smoke in either study. Increases in total inhaled mass were proportional to increasing exposure concentration in study 1, but in study 2 decreases in MV in groups exposed to 0.8- or 0.2-mg/L relative to groups exposed to 0.06 mg/L caused total inhaled mass for the high and middle dose groups to be lower in proportion to exposure concentration of smoke.

Inhalation exposure to smoke from test or reference cigarettes in both studies clearly induced microscopic changes in the nasal cavity, larynx, trachea, and lungs of exposed rats. Results of histopathologic examination of the recovery groups illustrated that these respiratory-tract lesions were either completely resolved or in the process of resolving by 13 wk after cessation of smoke exposure, and thus represent an adaptive response to the inhaled smoke. The nasal cavity and larynx were much more affected by inhaled smoke than the lungs in our studies, and the mucosal epithelium lining the base of the epiglottis and adjacent ventral pouch was the most affected site. The extreme susceptibility of the rodent laryngeal mucosa to inhaled smoke and other xenobiotics has been described in detail (Lewis, 1980, 1991; Gopinath et al., 1987; Burger et al., 1989). Since the most notable cellular changes observed in the respiratory tract of rodents in response to inhaled smoke involve cellular proliferation and metaplasia, a quantitative measure of cell turnover in affected tissue is a useful tool to measure the effect of exposure. Cell proliferation rate measurements in nasal turbinates and laryngeal epithelium using nuclear labeling with BrdU correlated well with histopathology data, reinforcing the conclusion that exposure to smoke from test or reference cigarette smoke for 13 wk clearly induced epithelial hyperplasia at these sites. Results of BrdU labeling in the trachea and lungs were less clear, and probably reflect the more subtle effects of inhaled smoke on the epithelium at these sites.

The effects of inhaled cigarette smoke on the respiratory tract of rats in both the studies described herein are similar to those described in a number of previously reported cigarette smoke inhalation studies in rats (Dalbey et al., 1980; Gaworski et al., 1997; Coggins et al., 1989; Ayres et al., 2001; Vanscheeuwijck et al., 2002) and hamsters (Lewis, 1980; Wehner et al., 1990). Four recently published papers have described studies similar to those presented here, in which smokes from cigarettes with and without flavoring or casing ingredients were compared on the basis of chemical composition and biologic effects on rodents (Gaworski et al., 1998; Paschke et al., 2002; Carmines, 2002; Baker et al., 2004). Results of the studies presented here are consistent with the conclusions of these authors that the presence of flavoring and casing ingredients studied to date did not significantly change the type or extent of toxicologic effects observed in rodents inhaling cigarette smoke.

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TCI AMERICA

SAFETY DATA SHEET

Revision number: 3
Revision date: 02/18/2017

1. IDENTIFICATION

Product name: 2-Hydroxy-3-methyl-2-cyclopentenone
Product code: H0469

Product use: For laboratory research purposes.
Restrictions on use: Not for drug or household use.

Company:
TCI America
9211 N. Harborgate Street
Portland, OR 97203 U.S.A.
Telephone:
+1-800-423-8616 / +1-503-283-1681
Fax:
+1-888-520-1075 / +1-503-283-1987
e-mail:
sales-US@TCIchemicals.com
www.TCIchemicals.com

Emergency telephone number:
Chemical Emergencies:
TCI America (8:00am - 5:00pm) PST
+1-503-286-7624
Transportation Emergencies:
Chemtrec 24-Hour
+1-800-424-9300 (U.S.A.)
+1-703-527-3887 (International)
Responsible department:
TCI America
Environmental Health Safety and Security
+1- 503-286-7624

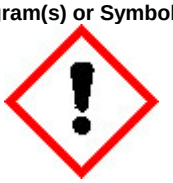
2. HAZARD(S) IDENTIFICATION

OSHA Haz Com: CFR 1910.1200: Acute Toxicity - Oral [Category 4]

Signal word: Warning!

Hazard Statement(s): Harmful if swallowed

Pictogram(s) or Symbol(s):



Precautionary Statement(s):

[Prevention]	Do not eat, drink or smoke when using this product. Wash hands and face thoroughly after handling.
[Response]	If swallowed: Immediately call a poison center or doctor. Rinse mouth.
[Storage]	None
[Disposal]	Dispose of contents and container in accordance with US EPA guidelines for the classification and determination of hazardous waste listed in 40 CFR 261.3. (See Section 13)

3. COMPOSITION/INFORMATION ON INGREDIENTS

Substance/Mixture: Substance
Components: 2-Hydroxy-3-methyl-2-cyclopentenone
Percent: >98.0%(T)
CAS Number: 80-71-7
Molecular Weight: 112.13
Chemical Formula: $C_6H_8O_2$
Synonyms: Cyclotene , 3-Methyl-1,2-cyclopentanedione , 3-Methyl-2-cyclopentenon-2-ol

4. FIRST-AID MEASURES

4. FIRST-AID MEASURES

Inhalation: Call a poison center or doctor if you feel unwell. Move victim to fresh air. Give artificial respiration if victim is not breathing. Administer oxygen if breathing is difficult. Keep victim warm and quiet. Treat symptomatically and supportively. Ensure that medical personnel are aware of the material(s) involved and take precautions to protect themselves.

Skin contact: Call a poison center or doctor if you feel unwell. Remove and isolate contaminated clothing and shoes. In case of contact with substance, immediately flush skin with running water for at least 20 minutes. Treat symptomatically and supportively. Ensure that medical personnel are aware of the material(s) involved and take precautions to protect themselves.

Eye contact: If this chemical contacts the eyes, immediately wash (irrigate) the eyes with large amounts of water, occasionally lifting the lower and upper eyelids. If eye irritation persists get medical advice/attention. Move victim to fresh air. Check for and remove any contact lenses. Keep victim warm and quiet. Treat symptomatically and supportively. Effects of exposure to substance may be delayed. Ensure that medical personnel are aware of the material(s) involved and take precautions to protect themselves.

Ingestion: Harmful if swallowed. If swallowed, seek medical advice immediately and show the container or label. Do not use mouth-to-mouth method if victim ingested the substance; give artificial respiration with the aid of a pocket mask equipped with a one-way valve or other proper respiratory medical device. Loosen tight clothing such as a collar, tie, belt or waistband. If a person vomits place them in the recovery position so that vomit will not reenter the mouth and throat. Rinse mouth. Keep victim warm and quiet. Treat symptomatically and supportively. Ensure that medical personnel are aware of the material(s) involved and take precautions to protect themselves.

Symptoms/effects:

Acute: No data available
Delayed: No data available

Immediate medical attention: WARNING: It might be hazardous to the person providing aid to give mouth-to-mouth respiration, because the inhaled material is harmful. If breathing has stopped, perform artificial respiration. Use first aid treatment according to the nature of the injury. Ensure that medical personnel are aware of the material(s) involved and take precautions to protect themselves.

5. FIRE-FIGHTING MEASURES

Suitable extinguishing media: Dry chemical, CO_2 , sand, earth, water spray or regular foam Consult with local fire authorities before attempting large scale fire fighting operations.

Specific hazards arising from the chemical

Hazardous combustion products: These products include: Carbon oxides
Other specific hazards: Closed containers may explode from heat of a fire.

Special precautions for fire-fighters:
Use water spray or fog; do not use straight streams. Dike fire-control water for later disposal; do not scatter the material. Containers may explode when heated. Move containers from fire area if you can do it without risk.
Special protective equipment for fire-fighters:
Wear positive pressure self-contained breathing apparatus (SCBA). Structural fire fighters' protective clothing provides limited protection in fire situations ONLY; it may not be effective in spill situations. Wear chemical protective clothing which is specifically recommended by the manufacturer. It may provide little or no thermal protection.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions: Avoid contact with skin, eyes, and clothing. Keep people away from and upwind of spill/leak. Do not touch damaged containers or spilled material unless wearing appropriate protective clothing (Section 8). Warn unnecessary personnel to move away. Stop leak if you can do it without risk. Ensure adequate ventilation. Isolate the hazard area and deny entry to unnecessary and unprotected personnel.

Personal protective equipment: Safety glasses. Lab coat. Dust respirator. Be sure to use a MSHA/NIOSH approved respirator or equivalent. Wear protective gloves (nitrile).

Emergency procedures: Prevent dust cloud. In case of a spill and/or a leak, always shut off any sources of ignition, ventilate the area, and excercise caution. Do not touch damaged containers or spilled material unless wearing appropriate protective clothing. Warn personnel to move away. Prevent entry into sewers, basements or confined areas; dike if needed.

Methods and materials for containment and cleaning up:
ELIMINATE all ignition sources (no smoking, flares, sparks, or flames in immediate area). Stop leak if without risk. Ventilate the area. Absorb with an inert material and put the spilled material in an appropriate waste disposal container. Use clean non-sparking tools to collect absorbed material.
Environmental precautions:
Keep away from living quarters. Prevent further leakage or spillage if safe to do so. Water runoff can cause environmental damage. Prevent entry into sewers, basements or confined areas; dike if needed.

7. HANDLING AND STORAGE

7. HANDLING AND STORAGE

Precautions for safe handling: Avoid inhalation of vapor or mist. Do not ingest. Avoid contact with skin and eyes. Good general ventilation should be sufficient to control airborne levels. Keep container dry. Handle and open container with care. Wear suitable protective clothing, gloves and eye/face protection. When using do not eat, drink, or smoke. Keep away from sources of ignition.

Conditions for safe storage: Keep only in the original container in a cool well-ventilated place. Keep away from incompatibles. Containers which are opened must be carefully resealed and kept upright to prevent leakage. Avoid prolonged storage periods.

Storage incompatibilities: Store away from oxidizing agents

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Exposure limits: No data available

Appropriate engineering controls:
Good general ventilation should be sufficient to control airborne levels. Ventilation is normally required when handling or using this product. Eyewash fountains should be provided in areas where there is any possibility that workers could be exposed to the substance. Follow safe industrial engineering/laboratory practices when handling any chemical.

Personal protective equipment

Respiratory protection: Dust respirator. Be sure to use a MSHA/NIOSH approved respirator or equivalent.
Hand protection: Wear protective gloves.
Eye protection: Safety glasses.
Skin and body protection: Lab coat.

9. PHYSICAL AND CHEMICAL PROPERTIES

Physical state (20°C): Solid
Form: Crystal - Powder
Color: White - Slightly pale reddish yellow
Odor: Characteristic
Odor threshold: No data available

Melting point/freezing point: 107°C (225°F)
Boiling point/range: No data available
Decomposition temperature: No data available
Relative density: No data available
Kinematic Viscosity: No data available

Partition coefficient: n-octanol/water (log P_{ow}) No data available

Flash point: 110°C (230°F)
Flammability (solid, gas): No data available

pH: No data available
Vapor pressure: No data available
Vapor density: No data available
Dynamic Viscosity: No data available

Evaporation rate: (Butyl Acetate = 1) No data available

Autoignition temperature: No data available
Flammability or explosive limits:
Lower: No data available
Upper: No data available

Solubility(ies):
Soluble: Methanol

10. STABILITY AND REACTIVITY

Reactivity: Not Available.
Chemical Stability: Stable under recommended storage conditions. (See Section 7)
Possibility of Hazardous Reactions: No hazardous reactivity has been reported.
Conditions to avoid: Avoid excessive heat and light.
Incompatible materials: Strong oxidizing agents
Hazardous Decomposition Products: No data available

11. TOXICOLOGICAL INFORMATION

RTECS Number: GY7298000

Acute Toxicity:
orl-gpg LD50:1400 mg/kg ipr-rat LDLo:500 mg/kg

Skin corrosion/irritation:
No data available

Serious eye damage/irritation:
No data available

Respiratory or skin sensitization:
No data available

Germ cell mutagenicity:
No data available

Carcinogenicity:

No data available

IARC: No data available **NTP:** No data available **OSHA:** No data available

Reproductive toxicity:
No data available

Routes of Exposure: Inhalation, Eye contact, Ingestion.
Symptoms related to exposure:
Overexposure may result in serious illness or death.
Potential Health Effects:
No specific information available; skin and eye contact may result in irriatation. May be harmful if inhaled or ingested.
Target organ(s): No data available

12. ECOLOGICAL INFORMATION

Ecotoxicity

Fish:	No data available
Crustacea:	No data available
Algae:	No data available

Persistence and degradability:	No data available
Bioaccumulative potential (BCF):	No data available
Mobillity in soil:	No data available
Partition coefficient: n-octanol/water (log P_{ow})	No data available
Soil adsorption (Koc):	No data available
Henry's Law: constant (PaM^3/mol)	No data available

13. DISPOSAL CONSIDERATIONS

Disposal of product: Recycle to process if possible. It is the generator's responsibility to comply with Federal, State and Local rules and regulations. You may be able to dissolve or mix material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber system. This section is intended to provide assistance but does not replace these laws, nor does compliance in accordance with this section ensure regulatory compliance according to the law. US EPA guidelines for Identification and Listing of Hazardous Waste are listed in 40 CFR Parts 261. The product should not be allowed to enter the environment, drains, water ways, or the soil.
Disposal of container: Dispose of as unused product. Do not re-use empty containers.
Other considerations: Observe all federal, state and local regulations when disposing of the substance.

14. TRANSPORT INFORMATION

DOT (US) Non-hazardous for transportation.

IATA Non-hazardous for transportation.

IMDG Non-hazardous for transportation.

15. REGULATORY INFORMATION

Toxic Substance Control Act (TSCA 8b.):
This product is ON the EPA Toxic Substances Control Act (TSCA) inventory.

US Federal Regulations

CERCLA Hazardous substance and Reportable Quantity:

SARA 313:	Not Listed
SARA 302:	Not Listed

State Regulations

State Right-to-Know

Massachusetts	Not Listed
New Jersey	Not Listed
Pennsylvania	Not Listed
California Proposition 65:	Not Listed

Other Information

NFPA Rating:

Health:	0
Flammability:	1
Instability:	0

HMIS Classification:

Health:	0
Flammability:	1
Physical:	0

International Inventories

WHMIS hazard class:	D2A: Materials causing other toxic effects. (Very Toxic)
Canada: DSL	On DSL
EC-No:	201-303-2

16. OTHER INFORMATION

Revision date: 02/18/2017
Revision number: 3
TCI chemicals are for research purposes only and are NOT intended for use as drugs, food additives, households, or pesticides. The information herein is believed to be correct, but does not claim to be all inclusive and should be used only as a guide. Neither the above named supplier nor any of its affiliates or subsidiaries assumes any liability whatsoever for the accuracy or completeness of the information contained herein. Final determination of suitability of any material is the sole responsibility of the user. All chemical reagents must be handled with the recognition that their chemical, physiological, toxicological, and hazardous properties have not been fully investigated or determined. All chemical reagents should be handled only by individuals who are familiar with their potential hazards and who have been fully trained in proper safety, laboratory, and chemical handling procedures. Although certain hazards are described herein, we can not guarantee that these are the only hazards which exist. Our SDS are based only on data available at the time of shipping and are subject to change without notice as new information is obtained. Avoid long storage periods since the product is subject to degradation with age and may become more dangerous or hazardous. It is the responsibility of the user to request updated SDS for products that are stored for extended periods. Disposal of unused product must be undertaken by qualified personnel who are knowledgeable in all applicable regulations and follow all pertinent safety precautions including the use of appropriate protective equipment (e.g. protective goggles, protective clothing, breathing equipment, face mask, fume hood). For proper handling and disposal, always comply with federal, state and local regulations.

Matrix Scientific

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Telephone: 803-788-9494 Fax: 803-788-9419

SAFETY DATA SHEET

Transportation Emergency: 3E Co. (5025) 800-451-8346

1. Product Identification

Name 3-Methylcyclopentane-1,2-dione
Catalog Number 127515
CAS Registry Number [765-70-8]
Company Matrix Scientific
Physical Address 131 Pontiac Business Center Drive
Elgin, SC 29045
USA
Telephone/Fax (803)788-9494/(803)788-9419

2. Hazard Identification

Hazardous Ingredients 3-Methylcyclopentane-1,2-dione

GHS label elements, including precautionary statements

Pictogram

Signal word WARNING

Hazard statement(s)

H317	May cause an allergic skin reaction
H319	Causes serious eye irritation

Precautionary statement(s)

P280	Wear protective gloves/protective clothing/eye protection/face protection.
P305+351+338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses if present and easy to do - continue rinsing.

3. Composition, Information or Ingredients

Name 3-Methylcyclopentane-1,2-dione

4. First Aid Measures

Eye Contact: Check for and remove any contact lenses. Immediately flush

	eyes with clean, running water for at least 15 minutes while keeping eyes open. Cool water may be used. Seek medical attention.
Skin Contact:	After contact with skin, wash with generous quantities of running water. Gently and thoroughly wash affected area with running water and non-abrasive soap. Cool water may be used. Cover the affected area with emollient. Seek medical attention. Wash any contaminated clothing prior to reusing.
Inhalation:	Remove the victim from the source of exposure to fresh, uncontaminated air. If victim's breathing is difficult, administer oxygen. Seek medical attention.
Ingestion:	Do NOT induce vomiting. Give water to victim to drink. Seek medical attention.

5. Fire-Fighting Measures

Extinguishing media:	Carbon dioxide, dry chemical powder, alcohol or polymer foam.
Special fire fighting procedures:	Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.
Unusual fire and explosion hazards/ decomposition of product:	Emits toxic fumes under fire conditions.

6. Accidental Release Measures

Steps to be taken if material is spilled or otherwise released into the environment - Wear Appropriate respirator, impervious boots and heavy rubber (or otherwise impervious) gloves. Scoop up solid material or absorb liquid material and place into appropriate container. Ventilate area and wash affected spill area after pickup is complete. Wash skin immediately with plenty of water. Place solid or absorbed material into containers and close for disposal.

7. Handling and Storage

Avoid prolonged exposure.
Use caution when handling.
Exposure to any chemical should be limited.
Do not breath dust or vapor.
Have safety shower and eye wash available.
Do not get in eyes, on skin or on clothing.
Keep container tightly closed.
Store in a cool, dry, well-ventilated place.
Ensure adequate ventilation during use.
Use only in a chemical fume hood.
To the best of our knowledge, the health hazards of this product have not been fully investigated.
This product is provided solely for the purpose of research and development.

8. Exposure Controls and Personal Protection

Wear Protective safety goggles.
Wear chemical-resistant gloves.
Wear protective clothing and chemical resistant boots.
Ensure ventilation during use.
After contact with skin, wash immediately.

9. Physical and Chemical Properties

Appearance: solid
Molecular Formula: C6H8O2
Molecular Weight: 112.13
Melting point (C): 104-108°

10. Stability and Reactivity

Incompatibilities: Strong oxidizing agents
Strong acids and bases

Hazard Decomposition Products

Carbon carbon monoxide
carbon dioxide

11. Toxicological Information

Acute effects:

Irritant
May be harmful by ingestion and inhalation.
Material is irritating to mucous membranes and upper respiratory tract.
To the best of our knowledge, the toxicological properties of this product have not been fully investigated or determined.

12. Ecological Information

Mobility: Data not known
Persistence and degradability: No data available
Cumulative potential: No data available
Other adverse effects: No data available

13. Disposal Considerations

Absent other actions demanded by federal or local regulations - Dissolve or mix the material with a combustible solvent and burn in a requlated, chemical incinerator equipped with after burner and scrubber.
Observe all federal, state and local laws.

14. Transport Information

Shipping Name Classed non-hazardous for shipment

15. Regulatory Information

Adhere to all Federal, State and local regulations.

16. Other Information

The information contained herein is accurate to the best of our knowledge, but is not meant to be complete and is included only as a guide. The end user is responsible for any damage resulting from handling or from contact with this product.