



Toxicological profile for 2,3,5,6-Tetramethylpyrazine

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

1. Name of substance and physico-chemical properties

1.1. IUPAC systematic name

2,3,5,6-Tetramethylpyrazine (PubChem).

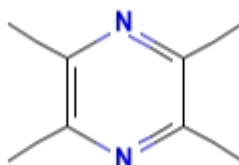
1.2. Synonyms

2,3,5,6-Tetramethylpyrazine; TETRAMETHYLPYRAZINE; Ligustrazine; Pyrazine, tetramethyl-; Tetrapyrazine; Tetramethylpyrazin; huanxiongzine; Liqustrazine; Ligustizine; 2,3,5,6-Tetramethyl pyrazine; chuanxingzine; FEMA No. 3237; 2,3,5,6,-Tetramethyl-1,4-pyrazine; Pyrazine, 2,3,5,6-tetramethyl-; 2,5,6-Tetramethylpyrazine; EINECS 214-391-2; Ligustrazin; 2,3,5,6-Tetramethylpyrazine; Pyrazine, 2,3,5,6-tetramethyl (PubChem)

1.3. Molecular formula

C₈ H₁₂ N₂ (PubChem)

1.4. Structural Formula



(PubChem)

1.5. Molecular weight (g/mol)

136.19 (PubChem)

1.6. CAS registration number

1124-11-4

1.7. Properties

1.7.1. Melting point

(°C): 77–86 (ChemSpider; EPISuite, 2017); 85-90 (FAO)

1.7.2. Boiling point

(°C): 190 (ChemSpider; EPISuite, 2017)

1.7.3. Solubility

7007–7010 mg/L at 25°C (estimated) (EPISuite, 2017); “slightly soluble in water” (FAO); <0.1 µg/mL (PubChem)

1.7.4. *pKa*

3.55 at 27°C (EPISuite, 2017)

1.7.5. *Flashpoint*

(°C): 128-130 (ChemSpider)

1.7.6. *Flammability limits (vol/vol%)*

No data available to us at this time.

1.7.7. *(Auto)ignition temperature*

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

1.7.8. *Decomposition temperature*

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

1.7.9. *Stability*

Stable under normal temperatures and pressures; Stable, incompatible with strong acids, strong oxidizing agents (ChemSpider)

1.7.10. *Vapor pressure*

0.147–0.169 mmHg at 25°C (estimated) (EPISuite, 2017); 0.7±0.4 mmHg at 25°C (estimated) (ChemSpider)

1.7.11. *log Kow*

1.28 (EPISuite, 2017); 0.564 (ChemSpider)

2. **General information**

2.1. *Exposure*

Used as a perfuming ingredient in cosmetics in the EU. As taken from CosIng (Cosmetic substances and ingredients database).

2,3,5,6-Tetramethylpyrazine is listed as a fragrance ingredient by International Fragrance Association (IFRA).

Based on the MSDI approach, estimated levels of intake as a flavouring substances in Europe and the US are 8 and 19 µg/person/day, respectively (EFSA, 2011; JECFA, 2002).

Reported levels from use as a flavouring (ppm): (FEMA, 1994).

Food category	Usual	Max.	Food category	Usual	Max.
Alcoholic beverages	0.26	1.03	Meat products	2.00	4.00
Baked goods	1.76	4.53	Nonalcoholic beverages	1.50	2.76

Frozen dairy	3.25	3.25	Soft candy	3.01	3.53
Gelatins, puddings	1.13	2.76			

Estimated intake from flavouring use: 0.00003248 mg/kg bw/day.

As taken from Burdock, 2010

2,3,5,6-Tetramethylpyrazine (CAS RN 1124-11-4) is used as a flavour enhancer in oral non-medicinal natural health products (Health Canada, 2021).

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%
2,3,5,6-tetramethylpyrazine	2136461080	99.53	1797130375	99.87
Total ion chromatogram	2146637628	100	1799431183	100

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

Ingredients CAS Number	Chemical Class	Mol. Wt. (M) Bp or Mp (°C)	Max cig Appln. Level (ppm)	Purity of sample Pyrolysed (%)	Composition of pyrolysate (Compound %)	Max level in smoke (nūig)
Tetramethylpyrazine CAS 1124-11-4	Pyrazine	M=136 Mp 85-90 Bp 190	20	98	Tetramethylpyrazine 100	10

2.3. Ingredient(s) from which it originates

Tetramethylpyrazine (TMP) is the major component extracted from the Chinese herb, Chuanxiong.

Occurs naturally in coffee 2.7 mg/kg; cocoa 0.2-33.5 mg/kg; defatted soya beans 0.01 mg/kg (CoE, 2000).

Occurs naturally in fried potato, bell pepper, wheat bread, Swiss cheese, Camembert, cooked beef, cooked lamb and mutton and liver, cooked uncured pork, beer, black and green tea, cocoa, coffee, dairy products, oatmeal, galbanum oil, roasted peanuts, soybean, beans, mushrooms, trassi, coriander seed, rice bran, sukiyaki, soy sauce, licorice, dried bonito, wild rice, shrimp, crab, clam, scallop, filberts, rum, sake, wine, whiskey, burley tobacco and soy products.

As taken from Burdock, 2010.

“[A] bacterial metabolite”.

As taken from PubChem.

3. Status in legislation and other official guidance

EFSA experts concluded that “The Panel agrees with the JECFA conclusion, “No safety concern at estimated levels of intake as flavouring substances” [8 and 19 µg/person/day in Europe and the US respectively], based on the MSDI approach (EFSA, 2011; JECFA, 2002).

2,3,5,6-Tetramethylpyrazine is included on the FDA’s inventory of “Substances Added to Food” (formerly EAFUS) as a flavoring agent or adjuvant. (FDA, 2024).

From use as a flavouring agent in food: 8 µg/person/day in Europe and 19 µg/person/day in the US (JECFA, 2002).

Not registered under REACH (ECHA).

2,3,5,6-Tetramethylpyrazine (CAS RN 1124-11-4) is not classified for packaging and labelling under Regulation (EC) No. 1272/2008 (ECHA, 2025).

2,3,5,6-Tetramethylpyrazine is authorised for use as a flavouring substance in all categories of flavoured food in the EU, under legislation (EU) no. 872/2012.

As taken from the European Commission, undated, Database of Food Flavourings.

Tetramethylpyrazine (CAS RN 1124-11-4) is listed in the US EPA Toxic Substances Control Act (TSCA) inventory and 2024 CDR TSCA Inventory list. The Chemical Data Reporting (CDR) Rule requires companies that manufacture (including import)

US EPA Substance Registry Services (SRS) – TSCA and CDR lists

2,3,5,6-Tetramethylpyrazine has been given GRAS (generally recognized as safe) status by FEMA (FEMA no. 3237) (Hall and Oser, 1970).

2,3,5,6-TETRAMETHYLPYRAZINE	
COE No.:	734
FEMA No.:	3237
JECFA No.:	780
Chemical names:	TETRAMETHYLPYRAZINE
Synonyms:	TETRAMETHYL-1,4-DIAZINE
Functional class:	FLAVOURING AGENT
Latest evaluation:	2001
ADI:	ACCEPTABLE
Comments:	No safety concern at current levels of intake when used as a flavouring agent
Report:	TRS 909-JECFA 57/51
Specifications:	COMPENDIUM ADDENDUM 9/FNP 52 Add.9/130
Tox monograph:	FAS 48-JECFA 57/135

JECFA (2003)

4. Metabolism/Pharmacokinetics

4.1. Metabolism/metabolites

The in vitro metabolism of 2,3,5,6-tetramethylpyrazine was reported at the 28th spring meeting of the German Society for Pharmacology and Toxicology (Hahnemann, 1987).

In this study TMP was used as a substrate of the cyt. P-460 in vitro. For this purpose it was incubated at a concentration of 2 mM with microsomes of control, PB- and MC-pretreated mice and

NADPH for 20 minutes at 37°C. HPLC-analysis of the methylcyanide-extracted incubation media on a reversed-phase-column revealed the existence of one single metabolite of TMP. After the synthesis of the two possible metabolites TMP-N-oxide (cf. Katrizky & Lagowski, 1971, Chem. Heterocyclic N-oxides, Acad. Press, London) and 2-hydroxymethyl-3,5,6-trimethylpyrazine (HO-TMP; cf. Houminer & Sanders, J. Heterocyclic Chem. 17, 647-649, 1980) the biologically formed metabolite could be identified by HPLC-methods as HO-TMP. It was not possible to detect the N-oxide in the incubation mixtures.

As taken from Hahnemann, 1987

4.2. Absorption, distribution and excretion

"The absorption of weak amine bases such as pyrazine derivatives is optimal at intestinal pH (5–7) (Schranker et al., 1957; Hogben et al., 1959). In humans and laboratory rodents, orally administered substituted pyrazines are rapidly absorbed from the gastrointestinal tract and excreted (Hawksworth & Scheline, 1975; Sjödin et al., 1989)."

As taken from JECFA, 2002.

"Tetramethylpyrazine permeated through buccal mucosa by a passive diffusion process." As taken from Liu C et al. Acta Pharmacol. Sin. 2002. 23(9), 792-6. PubMed, 2010

"The biotransformation of alkyl-, alicyclic-, and alkylaryl-substituted pyrazine derivatives is expected to occur by oxidation of the alkyl side-chains. Methyl-substituted pyrazines are oxidized to yield the corresponding pyrazine-2-carboxylic acids, while 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), which is a methyl- and ring-substituted pyrazine derivative, is oxidized to yield the corresponding hydroxymethyl derivatives (Turesky et al., 1988; Knize et al., 1989; Sjödin et al., 1989; Wallin et al., 1989). An alternative pathway for pyrazine derivatives involves hydroxylation of the pyrazine ring (Hawksworth & Scheline, 1975; Whitehouse et al., 1987; Yamamoto et al., 1987a,b)."

"Detoxication of alkyl-substituted pyrazines by side-chain oxidation and ring hydroxylation is comparable to the metabolic detoxication of alkyl-substituted pyridines in animals (Hawksworth & Scheline, 1975; Caputo et al., 1988; Blake & Beattie, 1989a; Caputo et al., 1989; Renberg et al., 1989; Oldham et al., 1990; Weidolf et al., 1992)."

"Products of oxidative metabolism can be excreted unchanged or conjugated with glycine, glucuronic acid, or sulfate before excretion (Caputo et al., 1989; Parkinson, 1996)."

"The elimination half-life of tetramethylpyrazine in rat blood and brain were 82.1 and 184.6 min, respectively." As taken from Tsai TH and Liang C. Int. J. Pharm. 2001. 216(1-2), 61-6. PubMed, 2010

The absorption of weak amine bases is optimal at intestinal pH. Orally administered substituted pyrazines are rapidly absorbed from the gastrointestinal tract and excreted (JECFA, 2002).

Excretion is via the urine; as both glucuronic acid conjugates and unchanged parent compound (JECFA, 2002).

"OBJECTIVE: To study the pharmacokinetics of single administration and different compatibility of tetramethylpyrazine (TMP) in rats. METHOD: Thirty two Sprague-Dawley rats were randomly divided into 4 groups: the TMP (30 mg x kg(-1)) group, the TMP+FA (30 mg x kg(-1) + 50 mg x kg(-1)) group, the TMP+TET (30 mg x kg(-1) mg x kg(-1)) group and the TMP+FA+TET (30 mg x kg(-1) + 50 mg x kg(-1) + 20 mg x kg(-1)) group. After the oral administration, their blood samples were collected to detect plasmas concentrations by HPLC method and to calculate pharmacokinetic parameters DAS 2.0 program. RESULT: In compatibility with FA, AUC(0-t), Cmax and Tmax showed no significant difference with the single administration of TMP, but t(1/2) and MRT,, were obviously longer than the single administration. In compatibility with TET and FA + TET, AUC (0-t),

C_{max} and T_{max} showed significant increase than the single administration of TMP, whereas t(1/2) and MRT₀, did not notably vary from the single administration. CONCLUSION: FA can prolong TMP's action time in rats, and TET can accelerate TMP's absorption in rats." As taken from Feng B et al. *Zhongguo Zhong Yao Za Zhi*. 2012. 37(9), 1311-4. PubMed, 2013

"A novel reservoir-type transdermal system of 2,3,5,6-tetramethylpyrazine (TMP) was developed containing eucalyptus oil as a penetration enhancer. The single and multiple-dose pharmacokinetic profiles of TMP administrated by TMP transdermal patch were characterized in healthy volunteers using an in vivo, randomized, open-label, two-way crossover design. 2,3,5,6-Tetramethylpyrazine phosphate (TMPP) oral tablets were chosen as reference. Following single/multiple oral administration of 200/100 mg TMPP tablets, a TMP C_{max} of 1284/613.5 ng/mL was observed within 0.75 h. Single/multiple applications of the TMP patch yielded mean C_{max} of 309/325 ng/mL at a median T_{max} of 5/4 h, with steady state achieved at second application. The mean C_{min} of the patch was 131±30.38 ng/mL, contrasting to nearly zero for the tablet. Multiple applications of patch produced an accumulative effect over single application. At steady state 250 mg/20 cm²) TMP patch given daily provided comparable exposure to 100 mg TMPP tablets three times daily (3753.91 versus 3563.67 ng·h/mL). TMP tablets and patch yielded similar steady-state plasma concentrations: C_{av} (148.48±51.27, 156.41±40.31 ng/mL). The results demonstrated that TMP patch can achieve a therapeutic effect that is comparable to oral administration, exhibited prolonged and sustained plasma levels, fewer drug fluctuations, lower adverse effects, more convenience, and improved patient compliance. In-vitro permeation through human skin demonstrated zero-order kinetics with the flux of 364 µg/cm²/h. The predicted C_{av} (163.9 ng/mL) was in agreement with the observed C_{av} (156.4 ng/mL)." As taken from Shen T et al. 2013. *Biol. Pharm. Bull.* 36(6), 931–7. PubMed, 2013

"The pharmacokinetic behaviors of tetramethylpyrazine (TMP) were investigated in rat plasma and microdialysates of various sites such as brain, blood and skin after tetramethylpyrazine hydrochloride (TMPH) transdermal or intraperitoneal (i.p.) administration. Samples were collected at timed intervals for the measurement of TMP by a quick and sensitive HPLC-UV method. The pharmacokinetic parameters were calculated by non-compartmental analysis using DAS 2.0. The results of pharmacokinetics indicated that the C_{max} in brain and plasma after transdermal administration (20 mg/cm², 1.2 cm²) was similar to that after i.p. administration (40 mg/kg). The value of C_{max} after i.p. administration in brain, blood microdialysates and plasma were 8.17 ± 2.06, 11.58 ± 2.66 and 15.54 ± 3.87 mg/l, respectively. After gel transdermal administration, the value of C_{max} in brain, blood, skin microdialysates and plasma were 7.29 ± 2.65, 8.53 ± 1.98, 43.39 ± 29.57 and 15.50 ± 2.99 mg/l, respectively. Compared with traditional administration, gel transdermal administration is a promising alternative to transport TMP to the brain." As taken from Tang Z et al. 2013. *Eur. J. Pharm. Sci.* 50(3-4), 454-8. PubMed, 2015

"The aim of this study was to quantify the oral delivery systems of Tetramethylpyrazine Microemulsion (TMP ME) to heart, liver, spleen, lung, kidney and brain by comparing TMP level after oral administration at a dose of 100 mg kg⁻¹) with those of TMP tablet suspension (TMP SWW). This study was taken in mice to develop a suitable analytical methodology in pharmacokinetics studies and to manipulate the tissue distribution and targeting evaluation. Drug concentrations in tissues were determined at different times post-mortem. An HPLC method for separation and quantification of TMP was developed and validated by studying mice tissues. Following oral administration, TMP concentrations in different tissues were constantly detected for quite a long time and finally differed significantly from each other. The AUC rank order 3 of ME group is AUC_{liver} > AUC_{brain} > AUC_{lung} > AUC_{spleen} > AUC_{heart} > AUC_{kidney}, while the SWW group is AUC_{liver} > AUC_{lung} > AUC_{spleen} > AUC_{heart} > AUC_{brain} > AUC_{spleen}. Especially, the AUC value in brain region (AUC_{brain}) of ME is 6.06-fold of SWW. The drug relative overall targeting efficiency (RTE) are calculated: heart (7.49%), liver (3.54%), spleen (12.60%), lung (6.02%), kidney (2.86%) and brain (12.51%). The results from ME directly showed obvious targeting transport to the brain. These results indicated that this new family of pharmaceutical

carriers can be used for the solubilization and targeted delivery of poorly soluble drugs to various pathological sites in the body". As taken from Ma L et al. 2013. Drug Dev. Ind. Pharm. 39(12), 1951-8. PubMed, 2015

"The objective of this article was to investigate the enhancing effect of menthone, menthol and pulegone on the transdermal absorption of drugs with different lipophilicity and probe their mechanisms of action at molecular level. Five model drugs, namely osthole, tetramethylpyrazine, ferulic acid, puerarin and geniposide, which were selected based on their lipophilicity denoted by logK_{ow}, were tested using in vitro permeation studies in which Franz diffusion cells and rat skin were employed. Infrared spectroscopy and molecular dynamic simulation were used to investigate the effect of these enhancers on the stratum corneum (SC) lipids, respectively. Three compounds could effectively promote the transdermal absorption of drugs with different lipophilicity, and the overall promoting capacities were in the following increasing order: pulegone < menthol < menthone. The penetration enhancement ratio was roughly in parabolic curve relationships with the drug lipophilicity after treatment with menthol or menthone, while the penetration enhancement effect of pulegone hardly changed with the alteration of the drug lipophilicity. The molecular mechanism studies suggested that menthone and menthol enhanced the skin permeability by disordering the ordered organization of SC lipids and extracted part of SC lipids, while pulegone appeared to promote drug transport across the skin only by extracting part of SC lipids." As taken from Lan Y et al. 2016. Pharm. Dev. Technol. 21(4), 389-98. PubMed, 2016

"In traditional Chinese medicine, *Angelica sinensis* is often coprescribed with *Ligusticum chuanxiong* Hort for the treatment of ischemic cerebrovascular diseases. Tetramethylpyrazine (TMP) is one of the most important active ingredients isolated from *Ligusticum chuanxiong* Hort; ferulic acid (FA) is the main water-soluble component of *Angelica sinensis*. 2. The purpose of this study is to investigate the possible effect of FA on the brain pharmacokinetics of TMP in conscious Sprague-Dawley rats. The pharmacokinetic parameters of TMP were investigated in brain microdialysates after oral and intravenous administration of TMP (4 mg/kg) to rats in the absence and presence of FA (5 mg/kg). Samples were collected at timed intervals for the measurement of TMP by a rapid and sensitive UPLC-MS/MS method. 3. The pharmacokinetic parameters were calculated by noncompartmental analysis for brain microdialysates. The brain pharmacokinetic data for TMP showed significant increases in C_{max}, t_{1/2}, AUC_{0-inf} and MRT_{0-inf} after combination with FA. After intragastric administration with FA, there were significant decreases in the T_{max} (from 38.33 ± 5.77 to 21 ± 5.48 min; p < 0.01) of TMP. This study indicated that potential drug-drug interaction between TMP and FA should be taken into consideration and the combined administration is beneficial in improving the bioavailability of TMP in the brain." As taken from Wu Y et al. 2017. Xenobiotica 47(11), 973-979. PubMed, 2018

"Tetramethylpyrazine (TMP) has been widely used in the treatment of ischemic cerebrovascular disease. However, the mechanism of TMP and how to increase its bioavailability need to be further explored. In our study, an in vivo microdialysis sampling technique coupled with ultra-performance liquid chromatography-mass spectrometry method was developed to investigate the pharmacokinetic properties of TMP and its interaction with different doses of borneol (BO) in rats. Linearity of TMP in brain and blood dialysates exhibited good linear relationships over the concentration range of 0.991-555.14 ng/mL. The specificity, linearity, accuracy, precision, matrix effect and stability were within acceptable ranges. The results demonstrated that BO had a marked impact on the pharmacokinetic properties of TMP. After co-administration, the areas under the concentration-time curve (AUC) of TMP in brain and blood were significantly increased. Meanwhile, the peak concentration of TMP in brain was also enhanced. The AUC_{Brain} /AUC_{Blood} of TMP, increased from 44% to 56 and 60.8% after co-administration with BO (15 and 30 mg/kg). The pharmacodynamic results showed that TMP co-administration with BO enhanced the cerebral blood flow during the period of ischemia and reduced the infarct volume. Overall, it might be an effective way to treat stroke to use TMP co-administered with BO." As taken from Liao W et al. 2018. Biomed. Chromatogr. 32(6), e4210. PubMed, 2019

“Shenxiong glucose injection (SXG) is a traditional Chinese medicine that is used for cardio-cerebral vascular diseases on the national essential drug list of China. To date, a comprehensive knowledge concerning the pharmacokinetic profile of SXG-related components, especially following multiple dosing, is still lacking. This study was designed to investigate the pharmacokinetics and tissue distribution of ligustrazine after single- and multiple-dose intravenous administration of SXG in rats. A simple HPLC method was developed for the determination of ligustrazine in biological samples. The pharmacokinetic profiles of ligustrazine in rats were linear after both single- and multiple-dose intravenous administration of SXG, with a half-life of approximately 35 min. Ligustrazine was readily distributed in highly perfused organs and almost eliminated from organs after 90 min of SXG injection. The AUC_{0-t} and C₀ of ligustrazine after SXG injection (18 ml/kg, equal to 9.0 mg/kg ligustrazine) were increased significantly compared to those of single ligustrazine administration (9.0 mg/kg), indicating that the pharmacokinetics of ligustrazine in the SXG were affected by other ingredients. This study provided first evidence for the pharmacokinetic characteristics of ligustrazine after both single and multiple-dose SXG in rats, which would be helpful for its clinical application.” As taken from Wang Q et al. 2019. *Naunyn Schmiedeberg's Arch. Pharmacol.* 392(5), 565-572. PubMed, 2020

4.3. Interactions

“OBJECTIVE: To observe the effects of Salvia combined with Ligustrazine on the TNF- α and TGF- β 1 in sera and bronchoalveolar lavage fluid (BALF) of rats with pulmonary fibrosis induced by bleomycin (BLM). METHODS: Male SD rats (n=90) were randomly assigned into 6 groups: normal saline (NS) group, BLM group, dexamethasone (DXM) group and three Chinese traditional herbal groups (C1: small dose group, C2: medium dose group, C3: large dose group). Rats were challenged intratracheally with BLM to establish pulmonary fibrosis rat models, while the NS group with 0.9% NaCl solution. Rats were given intra-peritoneal injections of normal saline (both the NS group and the BLM group), dexamethasone (the DXM group), or Salvia combined with Ligustrazine (the C1, C2, C3 groups). At the same time, 5 rats from the 6 groups were sacrificed at day 7, 14 and 28 after administration respectively. HE and Masson staining were performed to observe the extent of alveolitis and fibrosis; the levels of TNF- α and TGF- β 1 in both sera and BALF were detected by ELISA. RESULTS: Compared with NS group, a great deal of inflammatory cell infiltration into the alveolar was observed in BLM group at day 7, the alveolitis was lessened at day 14, while fibrosis was found; at day 28, the alveolitis became relieved and the fibrosis developed seriously. The extent of alveolitis and pulmonary fibrosis in DXM group was milder than that in BLM group at day 7, 14 and 28 ($P<0.05$). Compared with BLM group, the extent of pulmonary fibrosis in group C1 didn't remarkably change ($P>0.05$). The extent of alveolitis and pulmonary fibrosis in group C2 and C3 was significantly milder than that in BLM group at day 14, and the extent of pulmonary fibrosis in group C2 and C3 was remarkably reduced at day 28 ($P<0.05$). Compared with NS group, the levels of TNF- α and TGF- β 1 in both sera and BALF of the other groups were obviously higher ($P<0.05$). Compared with BLM group, the levels in DXM, C2 and C3 groups were lower at day 7, 14, 28 ($P<0.05$). Compared with DXM group, the levels in C1, C2 and C3 groups were lower at day 7, 14, 28 ($P<0.05$), and the difference between C2 and C3 groups was statistically significant ($P>0.05$). The levels of TNF- α and TGF- β 1 were consistent in rat sera and BALF. CONCLUSION: Medium and large doses of Salvia combined with Ligustrazine could abate the alveolitis and ameliorate the extent of pulmonary fibrosis by decreasing the levels of TNF- α and TGF- β 1 in both serum and BALF.” As taken from Huang C et al. 2013. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi.* 29 (7), 673–6. PubMed, 2013

“Ferulic acid (FA), ligustrazine (LZ) and tetrahydropalmatine (THP) are separately isolated from Chinese Angelica, Szechwan Lovage Rhizome and Rhizoma in the Jiawei-Foshou-San formula, a popular traditional Chinese medicine for irregular menses. It has been reported that the combination use of FA+LZ+THP has similar effect on endometriosis, but the underlying mechanisms are unclear. This study was to investigate the combination effects and mechanisms of

FA+LZ+THP on endometriosis rats. **MATERIALS AND METHODS:** Fifty endometriosis rats were intragastrically treated with FA+LZ+THP for 4 wk. The volume of ectopic endometrial tissue was measured to evaluate the effects. Then the changes in hypothalamic-pituitary-ovarian axis and ERE pathway were indicated by the levels of E2, GnRH, FSH and LH, and the expressions of ER, HSP90 and COX-2, respectively. In addition, peritoneal macrophages of each rat were cultured in vitro and treated with (FA+LZ+THP)-medicated serum for 24h. The proliferation and phagocytosis abilities, the levels of IL-1 β and TNF- α , and the expression of I κ B α were then measured for the changes of peritoneal macrophage activities. **RESULTS:** Combination use of FA+LZ+THP diminished the volume of the ectopic endometrial tissues ($P<0.05$ or $P<0.01$). It also decreased the E2 level, suppressed the expression of GnRH, FSH and LH, and decreased the protein expression of ER, HSP90 and COX-2 (all $P<0.05$ or $P<0.01$). The phagocytosis ability of peritoneal macrophage was enhanced by (FA+LZ+THP)-medicated serum ($P<0.05$) with no change of proliferation ($P>0.05$). Moreover, IL-1 β and TNF- α were downregulated (both $P<0.05$ or $P<0.01$) and I κ B α was upregulated by the (FA+LZ+THP)-medicated serum ($P<0.01$). **CONCLUSIONS:** The combination use of FA, LZ and THP could inhibit the growth of ectopic endometrial tissue in endometriosis rats. It might be related to the down-regulation of hypothalamic-pituitary-ovarian axis, the amelioration in ERE pathway and the improvement of peritoneal macrophage activities" As taken from Tang Q et al. 2014. *Ethnopharmacol.* 151(3), 1218-25. PubMed, 2015

"**OBJECTIVE:** To explore the effect of combination therapy of tetramethylpyrazine (TMP) with methotrexate (MTX) on collagen induced arthritis (CIA) rats. **METHODS:** Totally 55 male SD rats were stratified by body weight. Nine of them were randomly recruited as the normal control group. The rest 46 were immunized with type II bovine collagen (C II) for establishing rheumatoid arthritis (RA) model. Forty successfully modeled rats were randomly divided into 4 groups according to swollen toe degree, i.e., the CIA group, the TMP group, the MTX group, and the TMP plus MTX group, 10 in each group. Rats in the MTX group were administered with MTX (1.2 mg/kg), once per week for 4 continuous weeks. Those in the TMP group were administered with 40 mg/kg TMP, once per day for 10 continuous days, and then discontinued for 7 successive days, and continued for another 10 successive days. Rats in the TMP plus MTX group were administered with a mixture of equal dose MTX and TMP, and when MTX was discontinued, TMP was administered according to the way in the TMP group. Equal volume of saline solution was given to rats in the normal control group and the CIA group. Clinical parameters including ankle width (mediolateral diameter) and hindpaw swelling were measured at day 0, 4, 11, 18, and 26 after treatment. Rats were sacrificed 28 days after treatment, their knee joints and ankle joints were collected for pathological analyses. Serum levels of IL-1 β , IL-6, and IL-17A were detected by ELISA. Changes of fibrinogen (FIB) and platelet aggregation rate (PAg) were detected. **RESULTS:** Compared with the normal control group, the ankle width and hindpaw swelling increased significantly ($P<0.01$), contents of FIB and PAg increased obviously ($P<0.05$, $P<0.01$), serum levels of IL-1 β , IL-6, and IL-17 increased remarkably ($P<0.01$) in the CIA group. Obvious cell proliferation, inflammatory cell infiltration, hyperemia and edema of synovial tissues could be seen. Pannus formed and immersed in cartilages, resulting in necrosis. Compared with the model group, changes of ankle width and hindpaw swelling were all alleviated in each medicated group ($P<0.05$, $P<0.01$). Of them, the effect was superior in the MTX group to that of the TMP group and the MTX plus TMP group ($P<0.05$, $P<0.01$). Contents of FIB, serum levels of IL-1 β and IL-6 decreased significantly in the MTX group ($P<0.05$). Contents of FIB, serum levels of IL-1 β and IL-6 decreased significantly in the TMP group and the MTX plus TMP group ($P<0.05$). Besides, serum levels of FIB and IL-6 were obviously lower in the MTX plus TMP group than in the TMP group and the MTX group ($P<0.01$). Levels of PAg and IL-17A were more significantly lowered in the TMP group than in the MTX plus TMP group and the MTX group. Pathological changes could be alleviated in each medicated group, with the optimal effect obtained in the MTX plus TMP group. **CONCLUSION:** Combination of TMP with MTX could significantly ameliorate inflammatory reactions and FIB contents of CIA rats." As taken from Bian YQ et al. 2015. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 35(6), 724-9. PubMed, 2016

"The objective of this article was to investigate the enhancing effect of menthone, menthol and pulegone on the transdermal absorption of drugs with different lipophilicity and probe their mechanisms of action at molecular level. Five model drugs, namely osthole, tetramethylpyrazine, ferulic acid, puerarin and geniposide, which were selected based on their lipophilicity denoted by logKo/w, were tested using in vitro permeation studies in which Franz diffusion cells and rat skin were employed. Infrared spectroscopy and molecular dynamic simulation were used to investigate the effect of these enhancers on the stratum corneum (SC) lipids, respectively. Three compounds could effectively promote the transdermal absorption of drugs with different lipophilicity, and the overall promoting capacities were in the following increasing order: pulegone < menthol < menthone. The penetration enhancement ratio was roughly in parabolic curve relationships with the drug lipophilicity after treatment with menthol or menthone, while the penetration enhancement effect of pulegone hardly changed with the alteration of the drug lipophilicity. The molecular mechanism studies suggested that menthone and menthol enhanced the skin permeability by disordering the ordered organization of SC lipids and extracted part of SC lipids, while pulegone appeared to promote drug transport across the skin only by extracting part of SC lipids." As taken from Lan Y et al. 2016. Pharm. Dev. Technol. 21(4), 389-98. PubMed, 2016

"In traditional Chinese medicine, *Angelica sinensis* is often coprescribed with *Ligusticum chuanxiong* Hort for the treatment of ischemic cerebrovascular diseases. Tetramethylpyrazine (TMP) is one of the most important active ingredients isolated from *Ligusticum chuanxiong* Hort; ferulic acid (FA) is the main water-soluble component of *Angelica sinensis*. 2. The purpose of this study is to investigate the possible effect of FA on the brain pharmacokinetics of TMP in conscious Sprague-Dawley rats. The pharmacokinetic parameters of TMP were investigated in brain microdialysates after oral and intravenous administration of TMP (4 mg/kg) to rats in the absence and presence of FA (5 mg/kg). Samples were collected at timed intervals for the measurement of TMP by a rapid and sensitive UPLC-MS/MS method. 3. The pharmacokinetic parameters were calculated by noncompartmental analysis for brain microdialysates. The brain pharmacokinetic data for TMP showed significant increases in C_{max}, t_{1/2}, AUC_{0-inf} and MRT_{0-inf} after combination with FA. After intragastric administration with FA, there were significant decreases in the T_{max} (from 38.33±5.77 to 21±5.48 min; p<0.01) of TMP. This study indicated that potential drug-drug interaction between TMP and FA should be taken into consideration and the combined administration is beneficial in improving the bioavailability of TMP in the brain." As taken from Wu Y et al. 2017. Xenobiotica 47(11), 973-979. PubMed, 2018

"Tetramethylpyrazine (TMP) has been widely used in the treatment of ischemic cerebrovascular disease. However, the mechanism of TMP and how to increase its bioavailability need to be further explored. In our study, an in vivo microdialysis sampling technique coupled with ultra-performance liquid chromatography-mass spectrometry method was developed to investigate the pharmacokinetic properties of TMP and its interaction with different doses of borneol (BO) in rats. Linearity of TMP in brain and blood dialysates exhibited good linear relationships over the concentration range of 0.991-555.14 ng/mL. The specificity, linearity, accuracy, precision, matrix effect and stability were within acceptable ranges. The results demonstrated that BO had a marked impact on the pharmacokinetic properties of TMP. After co-administration, the areas under the concentration-time curve (AUC) of TMP in brain and blood were significantly increased. Meanwhile, the peak concentration of TMP in brain was also enhanced. The AUC_{Brain} /AUC_{Blood} of TMP, increased from 44% to 56 and 60.8% after co-administration with BO (15 and 30 mg/kg). The pharmacodynamic results showed that TMP co-administration with BO enhanced the cerebral blood flow during the period of ischemia and reduced the infarct volume. Overall, it might be an effective way to treat stroke to use TMP co-administered with BO." As taken from Liao W et al. 2018. Biomed. Chromatogr. 32(6), e4210. PubMed, 2019

"OBJECTIVE: To further explore the function of combine use of tetramethylpyrazine (TMP) and cisplatin (DDP) in lung carcinoma. METHODS: We used the combination drug to treat Lewis lung cancer mice, investigated the expression level of vascular endothelial growth factor (VEGF), Kruppel-like factor 4 (KLF4) and A disintegrin and metalloproteinase with thrombospondin motifs 1

(ADAMTS1) and to further explore the inhibitory effects and potential mechanism of TMP combined with DDP on tumor angiogenesis. RESULTS: The tumor growth was suppressed in TMP group, DDP group and TMP combined with DDP group. Furthermore, the weights and volume of tumor, the expression level of VEGF, KLF4 and ADAMTS1 were found significantly changed between experiment group and control group. These findings suggest that TMP with DDP had additional or synergistic effects to inhibit the tumor growth effectively, might be achieved through reducing the expression of angiogenesis promoting factor VEGF and increasing expression of angiogenesis inhibitors KLF4 and ADAMTS1. CONCLUSION: KLF4 and ADAMTS1 may be synergically involved in the angiogenesis in mouse Lewis lung cancer through the different signal ways." As taken from Tang JH et al. 2017. Asian Pac. J. Trop. Med. 10(8), 813-818. PubMed, 2018

"The present study aimed to determine the anticancer effects of the combination of β -elemene and ligustrazine in vitro as well as in vivo. Following evaluation using an MTT assay, β -elemene, ligustrazine and the β -elemene-ligustrazine combination treatments all exhibited the capacity to inhibit the growth of OS-732 cells, with inhibitory rates of 43.3, 54.4, and 75.0%, respectively. Using a flow cytometry assay, it was determined that the β -elemene-ligustrazine combination possessed the highest apoptotic rate (30.6%). Furthermore, β -elemene-ligustrazine combination treatment resulted in the highest downregulation of G protein-coupled receptor 124, vascular endothelial growth factor, matrix metalloproteinase (MMP)-2 and MMP-9 mRNA, and protein expression levels. In addition, the combined treatment led to an increase in the mRNA and protein expression of endostatin, TIMP metalloproteinase inhibitor (TIMP)-1 and TIMP-2 in OS-732 cells. Additionally, β -elemene-ligustrazine caused a decrease in nuclear factor- κ B, interleukin-8, C-X-C motif chemokine receptor 4 and urokinase-type plasminogen activator mRNA expression, as well as an increase in caspase-3, caspase-8, and caspase-9 mRNA expression. In vivo, the β -elemene-ligustrazine combination was able to reduce the weight and the bulk of the tumor in BALB/c-nu/nu nude mice compared with any other group. All the results described above regarding changes to mRNA and protein expression were further confirmed in vivo in the tumor tissue of mice. The results of the present study have suggested that the combination of β -elemene-ligustrazine exhibits greater anticancer effects compared with β -elemene- or ligustrazine-alone treatment." As taken from Fang M et al. 2018. Oncol. Lett. 15(3), 3957-3964. PubMed, 2018

"The purpose of this study was to determine the effects of different concentrations of ligustrazine, an extract from Chinese herb, on ketamine requirement for hypnosis and analgesia in mice. In the hypnotic response study, mice were randomly allocated to receive saline or ligustrazine at 10, 20, 40, 80 or 160 mg·kg⁻¹ by intraperitoneal injection. Ketamine was administered 15 min after ligustrazine injection. The hypnotic response was determined by assessing loss of the righting reflex (LORR) after ketamine injection. The dose of ketamine was determined by modified Dixon's up-and-down method in each group. In the analgesia study, different doses of ligustrazine were administered 15 min before 50 mg·kg⁻¹ ketamine injection. The analgesia effects (pain threshold) were determined by heat radiation-induced tail-flick latency and evaluated before ligustrazine administration or 5, 15, 30 and 60 min after ketamine administration. The ED₅₀ [95% confidence interval (CI)] for hypnosis induced by ketamine was 54.1 (44.8, 65.3) mg·kg⁻¹. Ligustrazine dose-dependently decreased the ED₅₀ for ketamine to induce hypnosis, which was [31.6 (26.2, 38.1)] mg·kg⁻¹ with the addition of 80 mg·kg⁻¹ ligustrazine and [27.7 (22.6, 33.7)] mg·kg⁻¹ with the addition of 160 mg·kg⁻¹ ligustrazine, respectively (p<0.05). Ligustrazine at 160 mg·kg⁻¹ also increased pain threshold in the presence of ketamine. Ligustrazine enhanced the hypnotic effect of ketamine in a dose-dependent manner. Ligustrazine at a large dose also increased the analgesic effect of ketamine." As taken from Liu C et al. 2018. Biol. Pharm. Bull. 41(5), 690-696. PubMed, 2019

"Rationale: Hepatic stellate cells (HSCs) are liver-specific pericytes regulating vascular remodeling during hepatic fibrosis. Here, we investigated how ligustrazine affects HSC pericyte functions. Methods: Rat HSC-T6 and human HSC-LX2 cells were cultured, and multiple molecular experiments including real-time PCR, Western blot, flow cytometry, immunofluorescence, electrophoretic mobility shift assay and co-immunoprecipitation were used to elucidate the

underlying mechanisms. Molecular simulation and site-directed mutagenesis were performed to uncover the target molecule of ligustrazine. Rats were intoxicated with CCl₄ for evaluating ligustrazine's effects in vivo. Results: Ligustrazine inhibited angiogenic cytokine production, migration, adhesion and contraction in HSCs, and activated PPAR γ . Selective PPAR γ inhibitor GW9662 potently abrogated ligustrazine suppression of HSC pericyte functions. Additionally, HIF-1 α inhibitor PX-478 repressed HSC pericyte functions, and ligustrazine inhibited the transcription of HIF-1 α , which was diminished by GW9662. Moreover, ligustrazine downregulation of HIF-1 α was rescued by knockdown of SMRT, and ligustrazine increased PPAR γ physical interaction with SMRT, which was abolished by GW9662. These findings collectively indicated that activation of PPAR γ by ligustrazine led to transrepression of HIF-1 α via a SMRT-dependent mechanism. Furthermore, molecular docking evidence revealed that ligustrazine bound to PPAR γ in a unique double-molecule manner via hydrogen bonding with the residues Ser289 and Ser342. Site-directed mutation of Ser289 and/or Ser342 resulted in the loss of ligustrazine transrepression of HIF-1 α in HSCs, indicating that interactions with both the residues were indispensable for ligustrazine effects. Finally, ligustrazine improved hepatic injury, angiogenesis and vascular remodeling in CCl₄-induced liver fibrosis in rats. Conclusions: We discovered a novel ligand activation pattern for PPAR γ transrepression of the target gene with therapeutic implications in HSC pericyte biology and liver fibrosis." As taken from Zhang F et al. 2018. *Theranostics* 8(3), 610-626. PubMed, 2018

"Puerarin (PUE) and tetramethylpyrazine (TMP) are central nervous system (CNS) drugs used in cerebrovascular diseases. Poor brain-blood barrier (BBB) permeability limited their clinical application. Borneol and α -asarone have been proposed as an oral brain-targeting enhancer. In this study, we aimed to first evaluate the 'orifice-opening' effect of borneol and α -asarone, both aromatic resuscitation drugs, on improvement of brain delivery of PUE and TMP and second to investigate whether the enhancing effects were associated with adenosine receptors (ARs)-mediated trans-BBB pathway. In vitro BBB model was established and borneol and α -asarone significantly increased the cumulative amount of permeated PUE and TMP and the enhancing effects could be counteracted by AR inhibitors. Borneol and α -asarone could decrease expression of ZO-1, an important BBB junction protein, but inversely increase the expression of A₁AR and A_{2A}AR. In vivo pharmacokinetic study also confirmed that oral co-administration of borneol or α -asarone significantly increased AUC_{brain} for PUE and TMP. These results suggested that borneol and α -asarone are both effective adjuvant agents for delivery of PUE and TMP to the brain." As taken from Wu JY et al. 2018. *Drug Deliv.* 25(1), 1858-1864. PubMed, 2019

"Borneol (BO) and menthol (MEN) are two widely used natural permeation enhancers in the transdermal drug delivery system. In previous studies, their permeation enhancement effects and mechanisms of action on the hydrophobic drug osthole (logP = 3.8) and hydrophilic drug 5-fluorouracil (logP = -0.9) have been studied. In this study, ligustrazine (LTZ), whose logP is 1.3, was used as a model drug to provide a comprehensive understanding of the influence of its logP on the permeation-enhancing effects of BO and MEN. Both BO and MEN enhanced the permeation of LTZ through the skin stratum corneum, as determined using the modified Franz diffusion cell experiment. The enhancement mechanisms were illustrated by coarse-grained molecular dynamics simulations as follows: at low concentrations, the enhancing ratio of MEN was higher than that of BO because of the stronger perturbation effects of MEN on the lipid bilayer, making it looser and facilitating LTZ diffusion. However, at high concentrations, in addition to the diffusion mechanism, BO induced the formation of water channels to improve the permeation of LTZ; however, MEN had no significant effects through this mechanism. Their results were different from those found with osthole and 5-fluorouracil and have been discussed in this study." As taken from Dai X et al. 2018. *Chem. Biol. Drug Des.* 92(5), 1830-1837. PubMed, 2019

"Recent published findings have demonstrated the effectiveness of combining molecules from traditional Chinese medicine with chemotherapeutic drugs to treat cancer. Combined administration of these agents can overcome drug-mitigating responses as well as reduce adverse side effects, thereby enhancing the efficacy of the therapy. Tetramethylpyrazine (TMP), an alkaloid monomer from the medicinal herb *Ligusticum chuanxiong* hort, is known to exert a variety of antitumor effects

including inhibition of tumor cell proliferation, metastasis, and drug resistance. In this research, we investigated antitumor effects of TMP combined with paclitaxel (PTX), a frontline chemotherapeutic drug, in vitro and in vivo. Our results indicate that TMP enhances the antitumor effects of PTX in ovarian cancer A2780 and SKOV3 cells. Furthermore, we found that combined treatment of TMP and PTX suppressed angiogenesis by inhibiting both ERK1/2 and Akt pathways and promoted apoptosis of tumor cells compared to TMP or PTX treatment alone. Moreover, TMP augmented the antitumor effects of PTX in ovarian cancer A2780 xenograft mouse models by significantly decreasing tumor burden and partially decreasing the toxicity of PTX, as evidenced by the decreased expression of proliferation and angiogenesis markers as well as the hematoxylin and eosin (H&E) staining and biochemical indexes assay. Overall, our findings provide novel mechanistic insight into the efficacy of combining of potent molecules present in traditional Chinese medicine with chemotherapeutic drugs for therapeutic intervention in cancer.” As taken from Zou L et al. 2019. *Front. Pharmacol.* 10, 707. PubMed, 2020

“Background: Leflunomide (LEF) is a first-line disease-modifying antirheumatic drug (DMARD) for rheumatoid arthritis (RA). However, there are still a few nonresponders. It is logical to suggest that employing combinations including LEF that produce synergistic effects in terms of pharmacological activity is a promising strategy to improve clinical outcomes. Methods: We propose a novel approach for predicting LEF combinations through investigating the potential effects of drug targets on the disease signaling network. We first constructed an RA signaling network with disease-associated driver genes. Thousands of available FDA-approved and investigational compounds were then selected based on a drug-RA network, which was generated using an algorithm model named synergistic score that combines chemical structure, functional prediction and target pathway. We then validated our predicted combination in a prospective clinical trial. Results: Ligustrazine (LIG), a key component of the Chinese herb Chuanxiong and an approved drug in China, ranked first according to synergistic score. In the clinical trial, after 48 weeks, the American College of Rheumatology (ACR) 20 response rate was significantly lower ($P < 0.05$) in the LEF group [58.8% (45.4%, 72.3%)] than in the LEF + LIG group [78.7% (68.5%, 89.0%)]. Consistently, the erosion score was lower in patients treated with LEF + LIG than in those treated with LEF (0.34 ± 0.20 vs 1.12 ± 0.30 , $P < 0.05$). Conclusions: Our algorithm combines structure and target pathways into one model that predicted that the combination of LEF and LIG can reduce joint inflammation and attenuate bone erosion in RA patients. To our knowledge, this study is the first to apply this paradigm to evaluate drug combination hypotheses.” As taken from Zhang C et al. 2019. *Chin. Med.* 14, 26. PubMed, 2020

“Bone marrow-derived mesenchymal stem cells (BMSCs) hold great promise for treating ischemic stroke owing to their capacity to secrete various trophic factors with potent angiogenic and neurogenic potentials. However, the relatively poor migratory capacity of BMSCs toward infarcted regions limits effective therapies for the treatment of stroke. The combination of BMSCs and pharmacological agent can promote the migration of BMSCs toward infarcted regions and improve the therapeutic effects after stroke. In this study, we aimed to investigate whether BMSCs combined with tetramethylpyrazine (TMP) enhanced BMSC migration into the ischemic brain, which had better therapeutic effect in the treatment of stroke. In a rat stroke model, we found that combination treatment significantly upregulated ischemic brain stromal-derived factor-1 (SDF-1) and CXC chemokine receptor 4 (CXCR4) expressions, and promoted BMSCs homing toward the ischemic regions than BMSC monotherapy. Moreover, BMSCs combined with TMP synergistically increased the expression of vascular endothelial growth factor and brain-derived neurotrophic factor, promoted angiogenesis and neurogenesis, and improved functional outcome after stroke. These results suggest that combination treatment could not only enhance the migration of BMSCs into the ischemic brain but also act in a synergistic way to potentiate endogenous repair processes and functional recovery after ischemic stroke.” As taken from Li L et al. 2019. *Stem Cells Dev.* 28(13), 871-881. PubMed, 2020

“The purpose of the study was to evaluate the effect of Astragalus membranaceus extract and ligustrazine combination on ameliorating inflammation in cerebral ischemic rats that have

undergone thrombolysis. Astragalus membranaceus and ligustrazine per se, or a combination of A. membranaceus and ligustrazine was administered by intraperitoneal injection immediately after surgery and sham surgery. After the induction of thrombolysis, the neurological function was measured and cerebral lesion volume was determined. The regulatory T cells in the spleen were measured by flow cytometry. To explore the protective effects of the combination drug on the neurological function and inflammation, the expression of transcription factor Foxp3 and cytokines, including transforming growth factor beta 1, interleukin 10, interleukin 4, interleukin 1 beta, interferon gamma, interleukin 17, in damaged brain was examined using reverse transcription polymerase chain reaction, Western blot, and enzyme-linked immunosorbent assay. The cerebral lesion volume was markedly reduced in the combination drug-treated rats compared to the rats treated with either A. membranaceus or ligustrazine alone ($P < 0.05$). The neurological function, regulatory T cells, transcription factor Foxp3, transforming growth factor beta 1, interleukin 10, and interleukin 4 were markedly elevated in the rats treated with combination drugs ($P < 0.05$). The expression of interleukin 1 beta, interferon gamma, and interleukin 17 was reduced in the rats treated with combination drug therapy ($P < 0.05$). Treatment with a combination of A. membranaceus and ligustrazine can ameliorate inflammation after thrombolysis and regulate the related cytokines by elevating the expression of endogenous regulatory T cells." As taken from Pan R et al. 2019. *Int. J. Immunopathol. Pharmacol.* 33, 2058738419869055. PubMed, 2020

"Purpose: To study the inhibitory effect of different doses of tetramethylpyrazine (TMP) combined with the beta-blocker, propranolol (Pro) on hemangioma endothelial (EOMA) cells. Methods: EOMA cells were cultured in vitro with varying doses of TMP and Pro (5, 10, 20 and 40 μ M). The effect of treatments on cell proliferation was assessed by MTT assay, while cell apoptosis was assayed by flow cytometry. The expressions of Bcl-2, Bax, p-mTOR, total-mammalian target of rapamycin (t-mTOR, p-p70S6) and total-p70 ribosomal protein S6 (t-p70S6) proteins were determined using Western blot. Results: MTT data showed that when used alone, TMP had no significant inhibitory effect on EOMA cells ($p > 0.05$). However, when TMP was combined with propranolol, there was significant inhibition of EOMA cells, and that the inhibition is dependent on TMP dose. Flow cytometry results showed that the combination of TMP and Pro induced EOMA cell apoptosis dose-dependently ($p < 0.05$). Moreover, TMP dose-dependently inhibited the phosphorylation of mTOR and p70S6 in EOMA cells, and enhanced Bax expression, but downregulated Bcl-2 ($p < 0.05$). Conclusion: These results suggest that TMP enhances the inhibitory influence of Pro p-mTOR and pp-70S6 in EOMA cells in a dose-dependent manner. Thus, TMP may enhance Pro-induced inhibition of the growth of endothelial cells, and promote apoptosis through suppression of activation of PI3K/AKT signal route. These findings provide a theoretical basis for the clinical application of TMP/Pro combination for the treatment of hemangioma." As taken from Jin W-W et al. 2019a. *Tropical Journal of Pharmaceutical Research* 18(4), 721-726.

"Context: Ligustrazine and valsartan are commonly used drugs in the treatment of cardiac and cardiovascular disease. Objective: The interaction between ligustrazine and valsartan was studied to investigate the effect of ligustrazine on the pharmacokinetics of valsartan. Materials and methods: The pharmacokinetics of valsartan (10 mg/kg) was investigated in Sprague-Dawley rats divided into three groups (with the pretreatment of 4 or 10 mg/kg/day ligustrazine for 10 days and without the pretreatment of ligustrazine as control) of six rats each. The in vitro experiments in rat liver microsomes were performed to explore the effect of ligustrazine on the metabolic stability of valsartan. Results: Ligustrazine changed the pharmacokinetic profile of valsartan. In the presence of 4 mg/kg ligustrazine, the AUC(0- t) (385.37 ± 93.05 versus 851.64 ± 104.26 μ g/L*h), $t_{1/2}$ (5.46 ± 0.93 versus 6.34 ± 1.25 h), and C_{max} (62.64 ± 9.09 versus 83.87 ± 6.15 μ g/L) of valsartan was significantly decreased, and the clearance rate was increased from 10.92 ± 1.521 to 25.76 ± 6.24 L/h/kg and similar changes were observed in the group with 10 mg/kg ligustrazine ($p < 0.05$). The metabolic stability of valsartan was also decreased by ligustrazine as the half-life of valsartan in rat liver microsomes decreased from 37.12 ± 4.06 to 33.48 ± 3.56 min and the intrinsic clearance rate increased from 37.34 ± 3.84 to 41.40 ± 4.32 μ L/min/mg protein ($p < 0.05$). Discussion and conclusions: Ligustrazine promoted the metabolism of valsartan via activating CYP3A4. The co-

administration of ligustrazine and valsartan should be taken into account.” As taken from Liu Y et al. 2020. Pharm. Biol. 58(1), 1290-1293. PubMed, 2021

“To investigate the effect of ligustrazine on the pharmacokinetic profile of tanshinol after intravenous administration in rats, a sensitive liquid chromatography tandem mass spectrometry method was developed and validated for quantitative determination of tanshinol and ligustrazine in rat plasma. After prepared by protein precipitation, the analytes were separated on a Waters Acquity HSS T3 column (100 × 2.1 mm, 1.8µm) and eluted by 0.1% formic acid in water and acetonitrile at a flow rate of 0.4 ml/min. The precursor-product ion transitions were m/z 197.0 → 135.0 for tanshinol, m/z 417.1 → 255.1 for liquiritin (internal standard) in negative ion mode and m/z 137.1 → 55.0 for ligustrazine in positive ion mode. To avoid the interference of tanshinol metabolite transformation, the stability of analytes in samples collected after administration was assessed. The validated method was successfully applied to a pharmacokinetic study after intravenous administration of single tanshinol and Danshen Chuanxiongqin Injection. After Danshen Chuanxiongqin injection administration, the values of elimination half-time, area under the concentration-time curve and Co were 0.36 ± 0.13 h, 1.29 ± 0.37 µg/ml h and 10.51 ± 2.58 µg/ml for male rats, respectively. In the single tanshinol group, the corresponding values were 0.56 ± 0.24 h, 1.85 ± 0.44 µg/ml h and 14.11 ± 2.26 µg/ml for male rats-30-40% higher than those for the Danshen Chuanxiongqin Injection group. There was a significant different between male and female rats. This study provided information on the influence of ligustrazine on the pharmacokinetic characteristics of tanshinol after intravenous administration of Danshen Chuanxiongqin Injection in rats, which will be helpful for its clinical application.” As taken from Jiao W et al. 2020. Biomed. Chromatogr. 34(9), e4869. PubMed, 2021

5. Toxicity

5.1. Single dose toxicity

Species	Route	Dose data	Reference
Rat	Oral	LD ₅₀ : 1910 mg/kg bw	DCTODJ Drug and Chemical Toxicology. (Marcel Dekker, 270 Madison Ave., New York, NY 10016) V.1- 1977/78- Volume(issue)/page/year: 3,249,1980
Mouse	Intraperitoneal	LD ₅₀ : 800 µg/kg bw	IJCREE International Journal of Crude Drug Research. (Swets & Zeitlinger B.V., POB 825, 2160 SZ Lisse, Netherlands) V.20- 1982- Volume(issue)/page/year: 23,119,1985
Mouse	Intravenous	LD ₅₀ : 239 mg/kg bw	CMJODS Chinese Medical Journal (Beijing, English Edition). (China International Book Trading Corp., POB 2820, Beijing, Peop. Rep. China) V.1- 1975- Adopted vol. no. 92 in 1979. Volume(issue)/page/year: 4,319,1978

As taken from RTECS, 2018.

2,3,5,6-Tetramethylpyrazine is suspected to be acutely toxic via the oral route. The Danish QSAR database contains information indicating that the substance is predicted as toxic via the oral route. As taken from ECHA, 2016.

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.

ECHA Annex III has been withdrawn as of April 2023: “The list of substances that are unlikely to benefit from the reduced information requirements, the so-called ‘Annex III inventory’, has been withdrawn.”

5.2. Repeated dose toxicity

“This tetra-alkyl-substituted pyrazines was tested in 90-day studies in rats treated in the diet. A control and a test group, each consisting of 15 male and 15 female albino weanling rats (Food and Drug Research Laboratories strain), were maintained individually in housing with controlled temperature and humidity and access to water and food ad libitum. The concentration of the test material in the diet was adjusted every 2 weeks to maintain a constant dietary intake of approximately 44 mg/kg bw per day of 2,3,5,6-tetramethylpyrazine. Clinical observations were recorded daily, and food consumption and body weights were determined weekly. During weeks 6 and 12 of the study, haematological, clinical chemical, and urinary parameters were measured on 10 animals of each sex. After 90 days, all animals were killed and subjected to detailed necropsy. Tissues and organs from each animal were preserved, and histopathological examinations were performed on major organs and tissues.

In female rats, daily dietary intake of 55 mg/kg bw per day of 2,3,5,6-tetramethylpyrazine resulted in slight to moderate decreases in body-weight gain (7–10%, not statistically significant) and statistically significant decreases in food use efficiency. No pathological lesions were found. No effects on body-weight gain or food use efficiency were reported in males fed 50 mg/kg bw per day of 2,3,5,6-tetramethylpyrazine. No other differences were found between test and control groups in either sex (Oser, 1969b,c,d,e).” As taken from JECFA, 2002.

Species	Route	Dose data	Toxic effects	Reference
Mouse	Intraperitoneal	TDL ₀ : 140 mg/kg/2W (intermittent)	Liver - other changes Kidney/Ureter/Bladder - other changes Endocrine - hypoglycemia	AJCHM* The American Journal of Chinese Medicine (Singapore ; River Edge, NJ : World Scientific Pub.) V.7-1979- Volume(issue)/page/year: 30,601,2002
Rat	Oral	TDL ₀ :5600 mg/kg/4W (intermittent)	Kidney/Ureter/Bladder - other changes Endocrine - hypoglycemia	PYTOEY Phytomedicine. (Gustav Fischer Verlag, Postfach 720143, D-70577 Stuttgart, Germany) V.1- 1994- Volume(issue)/page/year: 18,1148,2011

As taken from RTECS, 2021.

5.3. Reproduction toxicity

“Four groups of 10 virgin CrI:CD BR VAF Plus rats were given tetramethylpyrazine by gavage at a dose of 0, 25, 125, or 250 mg/kg bw per day 7 days before cohabitation and throughout cohabitation (maximum of 7 days), gestation, parturition, and a 4-day post-parturition period. The maternal indices monitored included daily measurement of body weight and food consumption, duration of gestation, and fertility parameters (mating, fertility, and gestation indices, number of offspring per litter). The indices in offspring included daily observations for clinical signs,

examination for gross external malformations, and measurement of body weight. The only effects reported were reduced body-weight gain in dams at the two higher doses, accompanied by a statistically significant reduction in food consumption in those at the highest dose. No effects were observed in dams at the lowest dose or in offspring at any dose. The authors concluded that tetramethyl-pyrazine had no reproductive or developmental effects (Vollmuth et al., 1997).” As taken from JECFA, 2002.

5.4. Mutagenicity

No data available to us at this time.

5.5. Cytotoxicity

“Tetramethylpyrazine and ferulic acid are two active ingredients of a Chinese herbal medicine *Ligusticum wallichii* Franchet. In the present investigation, iron-induced oxidative neuronal damage and the protective effects of tetramethylpyrazine and ferulic acid against this induction were studied in primary cultures of rat cerebellar granule cells. When neurons were treated with 200 microM of FeSO₄ for 1 h, lipid peroxidation in neurons increased time dependently, as measured with the thiobarbituric acid assay. Thirty-six hours after iron treatment, the cell viability decreased to 43.6% and the percentage of apoptotic cells increased to 50.6%. Transmission electron microscopic examination showed a disrupted nuclear envelope and condensed chromatin in iron-treated neurons. Analysis of DNA extracted from iron-treated cells by agarose gel electrophoresis showed the typical "ladder pattern", which indicated the formation of mono- and oligonucleosomes. After iron treatment, caspase 3 activity increased significantly, as measured in a fluoregenic assay. The results above suggested that iron treatment triggered oxidative stress and apoptosis in neurons. Western blot revealed that iron treatment up-regulated the apoptosis-related gene p53 as well as its effector gene p21(waf1/cip1). Pretreatment of the cells with 100 microM of tetramethylpyrazine or ferulic acid effectively decreased the activation of caspase 3 as well as the expression of p53 and p21(waf1/cip1), and attenuated iron-induced oxidative damage and apoptosis. The results suggest that tetramethylpyrazine and ferulic acid might be used as preventive agents against neuronal diseases associated with oxidative stress.” As taken from Zhang Z et al. Eur. J. Pharmacol. 2003. 467(1-3), 41-7. PubMed, 2010

“Activation of hepatic stellate cells (HSCs) is a pivotal event in the pathogenesis of liver fibrosis. Pharmacological induction of HSC apoptosis could be a promising strategy for fibrosis regression. Natural product tetramethylpyrazine (TMP) exhibits potent antifibrotic activities in vivo. However, the molecular mechanisms remain to be defined. The present study aimed at investigating the anti-proliferative and pro-apoptotic effects of TMP on HSCs and elucidating the underlying mechanisms. Our results demonstrated that TMP had no apparent cytotoxic effects on hepatocytes, but significantly inhibited HSC proliferation and induced cell cycle arrest at the G0/G1 checkpoint. These effects were associated with TMP regulation of cyclin D1, p21, p27 and p53. Furthermore, we found that TMP disrupted mitochondrial functions and led to activation of caspase cascades in HSCs. Mechanistic investigations revealed that TMP selectively blocked the extracellular signal-regulated kinase (ERK) signaling and activated p53, which was required for TMP induction of caspase-dependent mitochondrial apoptosis in HSCs. Autodock simulations predicted that TMP could directly bind to ERK2 with two hydrogen bonds and low energy score, indicating that ERK2 could be a direct target molecule for TMP within HSCs. Moreover, TMP altered expression of some marker proteins relevant to HSC activation. These data collectively revealed that TMP modulation of ERK/p53 signaling led to mitochondrial-mediated and caspase-dependent apoptosis in HSCs in vitro. These studies provided mechanistic insights into the antifibrotic properties of TMP that may be exploited as a potential option for hepatic fibrosis.” As taken from Zhang F et al. 2013. Apoptosis 18(2), 135–49. PubMed, 2013

"Tetramethylpyrazine (TMP) is an effective component of the traditional Chinese medicine Chuanxiong, which has been reported to have beneficial effects in various types of cancer. However, the activity and mechanism of action of TMP in osteosarcoma (OS) have not been elucidated to date. The aim of the present study was to investigate the inhibitory effect of TMP on OS and its underlying mechanism of action. OS cells were treated with various concentrations of TMP for 48 h. BALB/c nude mice with OS were treated with an intraperitoneal injection of TMP at a dose of 100 mg/kg every other day for 28 days. Cell proliferation was evaluated using an MTT assay. Cell cycle and apoptosis were measured using flow cytometry. The protein expression of nuclear and cytosolic nuclear factor- κ B (NF- κ B) p65, BCL-2 and cyclin D1 was measured using western blot analysis. TMP inhibited the proliferation of OS cells (MG-63, SAOS-2 and U2OS) in a dose-dependent manner. Additionally, TMP significantly induced apoptosis and G0/G1 arrest in MG-63 OS cells ($P < 0.05$). TMP upregulated the protein expression of cytosolic NF- κ B p65, while downregulating the protein expression of nuclear NF- κ B p65, BCL-2 and cyclin D1. Furthermore, TMP exerted a significant antitumor effect against OS in a xenograft tumor mouse model and exhibited a low toxicity. The present study provided fundamental evidence for the application of TMP in chemotherapy against OS." As taken from Wang Y et al. 2013. Mol. Med. Rep. 8(4), 984–8. PubMed, 2013

"Tetramethylpyrazine (TMPZ) is one of the active compounds extracted from the traditional Chinese medicinal herb Chuanxiong and several studies have shown it to possess anticancer properties. However, its effectiveness in gastric cancer and its cellular mechanisms are relatively unknown. The present study aimed to investigate the effect of TMPZ on SGC7901 cells, and it was demonstrated that a high dose of TMPZ inhibited cell viability and induced apoptosis by stimulating AMP-activated protein kinase (AMPK) through the generation of reactive oxygen species (ROS). Furthermore, TMPZ-induced apoptosis resulted in the sequential events beginning with the translocation of Bax, the collapse of mitochondrial membrane potential ($\Delta\Psi_m$), the release of cytochrome c and the activation of caspase-9 and -3. Each of these events was inhibited by compound C, a pharmacological inhibitor of AMPK. To the best of our knowledge, these results demonstrate for the first time that the induction of apoptosis by TMPZ in gastric cancer cells is associated with the activation of the ROS/AMPK pathway. AMPK activation induces apoptosis through the mitochondrial apoptotic pathway. In addition, these results raise the possibility that TMPZ may have a future therapeutic role in gastric cancer." As taken from Yi B et al. 2013. Oncol. Lett. 6(2), 583–589. PubMed, 2013

"AIM: To investigate the effects of tetramethylpyrazine (TMP) on proliferation and apoptosis of the human gastric carcinoma cell line 7901 and its possible mechanism of action. METHODS: The viability of TMP-treated 7901 cells was measured with a 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) and cell apoptosis was analyzed by flow cytometry. The distribution of cells in different phases of cell cycle after exposure of TMPs was analyzed with flow cytometry. To investigate the molecular mechanisms of TMP-mediated apoptosis, the expression of NF- κ Bp65, cyclinD1 and p16 in SGC-7901 cells was analyzed by reverse transcription- polymerase chain reaction (RT-PCR) and western blotting. RESULTS: TMP inhibited the proliferation of human gastric carcinoma cell line 7901 in dose and time dependent manners. Cell growth was suppressed by TMP at different concentrations (0.25, 0.5, 1.0, 2.0 mg/ml), the inhibition rate is 0.46%, 4.36%, 14.8%, 76.1% (48h) and 15.5%, 18.5%, 41.2%, 89.8% (72h) respectively. When the concentration of TMPs was 2.0mg/ml, G1-phase arrest in the SGC-7901 cells was significant based on the data for cell cycle distribution. RT-PCR demonstrated that NF- κ Bp65 and cyclin D1 mRNA expression was significantly down-regulated in 7901 cells treated with 2.0 mg/ml TMP for 72h ($P < 0.05$), while the p16 mRNA level was up-regulated ($P < 0.05$). The protein expression of NF- κ Bp65 and cyclin D1 decreased gradually with the increase in TMP concentration, compared with control cells ($P < 0.05$), while expression of protein p16 was up-regulated ($P < 0.01$). CONCLUSION: TMP exhibits significant anti-proliferative and pro-apoptotic effects on the human gastric carcinoma cell line SGC-7901. NF- κ Bp65, cyclinD1 and p16 may also play important roles in the regulation mechanisms". As taken from Ji AJ et al. 2014. Asian Pac. J. Cancer Prev. 15(8), 3581-6. PubMed 2015

"Ligustrazine has been used to treat heart and blood vessel disease in China. In the present study, we investigated the potential action of Ligustrazine as a component of chuanxiong (a Chinese herb) in scavenging hydroxyl radical and superoxide radical as indicated in the ESR spin-trapping measurement. Treatment of Ligustrazine in mice decreased mortality after whole body γ -irradiation. The anti-radiation action of Ligustrazine was studied by measuring DNA damage (Comet assay and γ -H2AX formation) and apoptosis induced by irradiation. It was triggered by altering the level of DNA-PKcs protein, a critical component of DNA double-strand break (DSB) repair pathways in mice after irradiation. Consistently, the phosphorylation of Akt protein, a mediator of survival signaling, was concurrently increased by Ligustrazine treatment. Additionally, the cytokines along with the phosphorylation of the p38 protein which is activated by a variety of environmental stresses and inflammatory cytokines decreased in the Ligustrazine-treated group as compared to irradiation group. Our results suggest that Ligustrazine has radioprotective effect through its capabilities as a powerful antioxidant, in reducing reactive oxygen species (ROS) level induced by irradiation, minimizing DNA damage and apoptosis, and activating survival signal Akt pathways. This study will be of value in the development of novel radioprotective compounds". As taken from Zheng H et al. 2013. *Environ. Toxicol. Pharmacol.* 36(3), 1197-206. PubMed, 2015

"Hepatocellular carcinoma (HCC) is one of the most common types of liver cancers with high recurrence rate and mortality rate. Recent studies have indicated that tetramethylpyrazine (TMP), a purified chemical extracted from *Ligusticum wallichii* Franchet (ChuanXiong), possessed antitumor effects on HCC, but detailed mechanism remains unclear. Our study aims at investigating the antitumor effect of TMP on HCC and its underlying mechanism. We found that TMP inhibited cell proliferation of HepG2 cells in a dose-dependent way, and xenograft tumor models also indicated that high concentrations of TMP administration inhibited tumor growth. Next, flow cytometric analysis and transmission electron microscope images showed that TMP enhanced cell apoptosis in HepG2 cells, and western blot results showed that TMP promoted cleavage of caspase-3 and PARP in vitro and in vivo. We also found that TMP caused autophagy in HCC in vitro and in vivo. In order to examine the role of autophagy in TMP-induced apoptosis, 3-methyladenine (3-MA) was used to block the action of autophagy. Our data showed TMP-induced autophagy might be a pro-apoptosis process in HCC. Furthermore, the results of anti-oxidative enzymes and oxidation-sensitive fluorescent probe 2, 7-dichlorofluorescein diacetate (DCFH-DA) indicated that TMP induced ROS generation and inhibition of ROS diminished the anticancer function of TMP. In conclusion, our studies provide new insights into the mechanisms underlying the antitumor effect of TMP and suggest that TMP can be a novel therapeutic regimen for HCC." As taken from Cao J et al. 2015a. *Int. Immunopharmacol.* 26(1), 212-20. PubMed, 2016

"Hepatocellular carcinoma (HCC) is the fifth most common cancer. An important approach to control HCC is chemoprevention. This study aims at investigating the antitumor effect of Tetramethylpyrazine (TMP). Rats were injected with N-Nitrosodiethylamine (DEN) to establish HCC. Tumor development was observed. Liver function was evaluated. Apoptosis and cell cycle arrest-related makers and signaling cascades were determined by Western blot, RT-PCR and flow cytometric analysis. The administration of TMP could significantly inhibit tumor development in DEN-induced HCC rats, shown by reduced incidence of tumor, decreased number of tumor nodules and reduced maximal size of tumor. DEN-induced increase of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase and alkaline phosphatase activities were significantly inhibited by TMP. TMP exhibited inhibitory effect on HCC through induction of apoptosis and cell cycle arrest in rats. TMP induced apoptosis through increasing Bax, decreasing Bcl-2, increasing the release of cytochrome c, and activating caspase, which consisted of the mitochondrial apoptotic pathway. TMP induced G2/M cell cycle arrest through down-regulation of cyclin B1/cdc2. In addition, inhibition of Akt and ERK signaling and the antioxidant activities of TMP may also contribute to its antitumor effect. These data provide new insight into the mechanisms underlying the antitumor effect of TMP." As taken from Cao J et al. 2015b. *J. Biol. Regul. Homeost. Agents* 29(2), 297-306. PubMed, 2016

"OBJECTIVE: To study the proliferation and apoptosis of tetramethylpyrazine (TMP) on leukemic U937 cells and its possible mechanism. METHOD: The inhibitory effect of TMP on the proliferation of U937 cells was detected by CCK-8 assay. The cell apoptosis and cycle distribution were examined by the flow cytometry. The mRNA expressions of bcl-2 and P27 were determined by the Real-time PCR. Western blot was carried out to detect bcl-2, caspase-3, cyclin E1, CDK2 and P27 expressions. RESULT: TMP inhibited the proliferation of U937 cells in a dose-and-time dependent manner, with IC50 value of 160 mg x L(-1) at 48 h. In addition, TMP could induce the apoptosis of U937 cells and block the cell cycle in G0/G1 phase. According to the results of Real-time PCR and Western blot, TMP could down-regulate the expression of apoptosis-related molecule bcl-2, cycle-related protein cyclin E1 and CDK2 and up-regulate caspase-3 and P27. CONCLUSION: TMP shows the effects in inhibiting the proliferation of leukemic U937 cells and inducing the apoptosis. Its mechanism may be related to the impacts on the cell cycle distribution, down-regulation of the bcl-2 expression, which finally activates caspase-3, starts the apoptosis path and causes the cell apoptosis." As taken from Wang XJ et al. 2015a. Zhongguo Zhong Yao Za Zhi 40(11), 2186-90. PubMed, 2016

Tetramethylpyrazine (TMP) has been proven to be an anticancer agent in many studies. However, its effectiveness in acute lymphoblastic leukemia (ALL) and its molecular mechanisms are still unclear. The present study aimed to evaluate the effect of TMP against Jurkat and SUP-B15 ALL cell lines and to investigate the possible detailed mechanism of action of TMP. A Cell Counting Kit-8 (CCK-8) assay was employed to examine the proliferation of Jurkat and SUP-B15 cells. Flow cytometric analysis was conducted to detect the cell cycle distribution and apoptotic rate. The expression of total glycogen synthase kinase-3 β (GSK-3 β), cox-2, survivin, bcl-2 and p27 RNA and protein levels was detected by quantitative real-time PCR and western blot assay, respectively. Additionally, western blot analysis was used to determine the whole-cell and nuclear protein levels of GSK-3 β downstream transcription factors, NF- κ B (p65) and c-myc. TMP inhibited the proliferation of Jurkat and SUP-B15 cells in a dose- and time-dependent manner, with IC50 values of 120 and 200 μ g/ml, respectively at 48 h. TMP induced the apoptosis of Jurkat and SUP-B15 cells and synergistically blocked cell cycle progression at the G0/G1 phase. Cells treated with TMP exhibited significantly attenuated GSK-3 β , NF- κ B (p65) and c-myc expression, followed by downregulation of bcl-2, cox-2 and survivin and an upregulation of p27. The results showed that TMP induced apoptosis and caused cell cycle arrest in Jurkat and SUP-B15 cells through the downregulation of GSK-3 β , which may have further prevented the induced translocation of NF- κ B and c-myc from the cytoplasm to the nucleus." As taken from Wang XJ et al. 2015b. Oncol. Rep. 33(5), 2368-74. PubMed, 2016

"Tetramethylpyrazine (TMP), one of the active ingredients isolated from a Chinese herbal prescription, possesses protective effects against apoptosis in endothelial cells. However, the underlying mechanism of its protective effects in endothelial cells remains to be elucidated. Using human umbilical vein endothelial cells (HUVECs), the present study assessed the protective effects of TMP on CoCl2-induced apoptosis. Following pre-incubation with CoCl2 (150 μ M/ml) for 4 h, the HUVECs were treated with TMP at different concentrations (50, 100 and 200 μ M/ml) for 8 h. TMP upregulated the expression of prolyl hydroxylase (PHD)2, reduced the protein and mRNA expression levels of vascular endothelial growth factor (VEGF), and reduced the expression of HIF-1 α only at the protein level, not at the mRNA level in HUVECs, in a concentration-dependent manner. Furthermore, silencing of the PHD2 gene with small interfering (si)RNAs abolished the reduction in the expression of hypoxia-inducible factor (HIF)-1 α and VEGF by TMP. In addition, TMP protected CoCl2-induced HUVEC injury via an apoptosis pathway, as characterized by the increased ratio of cell viability and the reduced percentage of apoptotic and terminal deoxynucleotidyl transferase dUTP nick end labeling-positive HUVECs, activation of caspase-3, -8 and -9, B-cell lymphoma (Bcl)-2/Bcl-2-activated X protein expression, as well as the release of cytochrome c. The protective properties of TMP were partially attributed to the mRNA and protein expression levels of PHD, since silencing of the PHD2 gene with siRNAs abolished these effects. The present study demonstrated that the antiapoptotic effect of TMP in CoCl2-induced HUVECs

was, at least in part, via the regulation of the PHD2/HIF-1 α signaling pathway.” As taken from Yang C et al. 2016. Mol. Med. Rep. 13(2), 1287-96. PubMed 2016

“To study the effects of tetramethylpyrazine (TMP) on the proliferation of hepatic stellate cells-T6 (HSC-T6), and the expression of connective tissue growth factor (CTGF) and Smad2/3 in these cells, HSC-T6 cells were cultured with TMP at different concentrations after transforming growth factor- β 1 (TGF- β 1) stimulation. MTT assay was used to assess the cell proliferation. Cells were divided into the control group, TGF- β 1-treated group and TMP-treated groups, which were treated with different concentrations of TMP. Immunocytochemistry and western blot were performed to detect the expression levels of CTGF and Smad2/3 in HSC-T6 cells. MTT analysis indicated that TMP significantly inhibited the proliferation of HSC-T6 cells, in dose-dependent and time-dependent manners. Immunocytochemistry detection and western blot showed that TMP could diminish TGF- β 1-induced CTGF over-expression in HSC-T6 cells. Similarly, the enhancing effects of TGF- β 1 on Smad2/3 expressions in HSC-T6 cells could also be counteracted by TMP treatment. Nuclear translocation of Smad2/3 was blocked by TMP treatment. Correlation analysis suggested a positive correlation between CTGF and Smad2/3 expression levels in HSC-T6 cells. TMP exerts anti-hepatic fibrosis effect through decreasing the expression of CTGF and Smad2/3, as well as inhibiting the proliferation of HSC-T6 cells. Our study provides cellular and molecular bases for further application of TMP in the clinical treatment for hepatic fibrosis.” As taken from Li J et al. 2015. Biotechnol. Biotechnol. Equip. 29(1), 124-131. PubMed, 2016

“BACKGROUND AND AIM: Tetramethylpyrazine (TMP), a major alkaloid isolated from Ligusticum chuanxiong, has been reported in hepatic fibrosis models. However, the action mechanism remains unclear. In the present study, effects of tetramethylpyrazine (TMP) against hepatic stellate cell (HSC) activation as well as the possible mechanisms were evaluated. METHODS: Western blot assay was used to detect TMP effects on protein expression of Smo, Patched, Hhip, and Gli and to investigate the effects of TMP on Cyclin D1, Cyclin E1, CDK2, Bcl-2, Bax, and caspase expression with cyclopamine supplementation. RESULTS: Our results showed that TMP significantly inhibits the expression of Cyclin D1, Cyclin E1, and Cyclin-dependent kinase CDK2 and changes the HSC cycle by inhibiting the proliferation of HSC. Moreover, TMP has also been shown to decrease the expression of Bcl-2 and increase the expression of Bax in HSC-T6 cells. Furthermore, TMP can inhibit the expression of connective tissue growth factor (CTGF), and the inhibitory effect was intensified after the application of joint treatment with TMP and cyclopamine. CONCLUSION: TMP may be an effective Hh signaling pathway inhibitor for hepatic fibrosis treatment.” As taken from Hu J et al. 2015a. Biomed. Res. Int. 2015, 603067. PubMed, 2016

“Tetramethylpyrazine (TMP) is a compound purified from herb. Its effect on Ca²⁺ concentrations ([Ca²⁺]_i) in renal cells is unclear. This study examined whether TMP altered Ca²⁺ signaling in Madin-Darby canine kidney (MDCK) cells. TMP at 100-800 μ M induced [Ca²⁺]_i rises, which were reduced by Ca²⁺ removal. TMP induced Mn²⁺ influx implicating Ca²⁺ entry. TMP-induced Ca²⁺ entry was inhibited by 30% by modulators of protein kinase C (PKC) and store-operated Ca²⁺ channels. Treatment with the endoplasmic reticulum Ca²⁺ pump inhibitor 2,5-di-tert-butylhydroquinone (BHQ) inhibited 93% of TMP-evoked [Ca²⁺]_i rises. Treatment with TMP abolished BHQ-evoked [Ca²⁺]_i rises. Inhibition of phospholipase C (PLC) abolished TMP-induced responses. TMP at 200-1000 μ M decreased viability, which was not reversed by pretreatment with the Ca²⁺ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester. Together, in MDCK cells, TMP induced [Ca²⁺]_i rises by evoking PLC-dependent Ca²⁺ release from endoplasmic reticulum and Ca²⁺ entry via PKC-sensitive store-operated Ca²⁺ entry. TMP also caused Ca²⁺-independent cell death.” As taken from Fang YC et al. 2017. J. Biochem. Mol. Toxicol. 31(10), e21952. PubMed, 2018

Type of Test	Route of Exposure or Administration	Species/Test System	Dose Data	Toxic Effects	Reference

ICLo - Inhibitor Concentration Low	In vitro	Human - liver tumor	1.5 gm/L/24H	In Vitro Toxicity Studies - cell viability (cell death), unspecified assay	FCTOD7 Food and Chemical Toxicology. (Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, NY 10523) V.20- 1982- Volume(issue)/page/year: 109,930,2017

As taken from RTECS, 2021

High-throughput Assay Data

The US Environmental Protection Agency (EPA) evaluated tetramethylpyrazine in a series of high-throughput assays, which are publicly available on the US EPA's CompTox Dashboard (section BIOACTIVITY / sub-section TOXCAST:SUMMARY), available at the following URL: <https://comptox.epa.gov/dashboard>

US EPA provides the following data use considerations for ToxCast data: "The activity of a chemical in a specific assay does not necessarily mean that it will cause toxicity or an adverse health outcome. There are many factors that determine whether a chemical will cause a specific adverse health outcome. Careful review is required to determine the use of the data in a particular decision contexts. Interpretation of ToxCast data is expected to change over time as both the science and analytical methods improve."

A summary of the ToxCast assay data on tetramethylpyrazine is provided below in Figure 1. Figure 1 proves an overview of the types of assays where activity was noted with this substance. The complete study details are available on US EPA's CompTox Dashboard.

Figure 1



“Sensorineural hearing loss is prevalent in patients receiving cisplatin therapy. Tetramethylpyrazine (Tet) and tanshinone IIA (Tan IIA) have protective roles against hearing impairment or ototoxicity. The present study aimed to investigate the molecular mechanisms underlying cisplatin-induced ototoxicity and the protective effect of Tet and Tan IIA against it. House Ear Institute-Organ of Corti 1 auditory cells were treated with titrating doses of Tan IIA, Tet, and cisplatin. In a cell viability assay, cisplatin, Tan IIA and Tet had IC₅₀ values of 42.89 μ M, 151.80 and 1.04x10³ mg/l, respectively. Tan IIA augmented cisplatin-induced cytotoxicity. However, Tet concentrations <75 mg/l attenuated cisplatin-induced cytotoxicity and apoptosis. Moreover, RNA sequencing analysis was carried out on auditory cells treated for 30 h with 30 μ M cisplatin alone for 48 h or combined with 37.5 mg/l Tet for 30 h. Differentially expressed genes (DEGs) induced in these conditions were identified and examined using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis. Cisplatin increased the expression of genes related to the p53 and FoxO pathways, such as Fas, p21/CDKN1A, and Bcl-2 binding component 3, but decreased the expression of insulin-like growth factor 1 (IGF1), as well as genes in the histone (Hist)1 and Hist2 clusters. Treatment with Tet downregulated FOXO3 and Bcl-2 binding component 3, and increased the expression of IGF1. Moreover, Tet upregulated genes associated with Wnt signaling, but not p53-related genes. Thus, the otoprotective properties of Tet might be mediated by activation of Wnt and IGF1 signaling, and inhibition of FoxO signaling.” As taken from Guan G et al. 2020. Mol. Med. Rep. 22(6), 5052-5068. PubMed, 2021

“Chuanxiong Rhizoma, the dried rhizome of *Ligusticum chuanxiong* Hort., is a commonly used drug for promoting blood circulation and dissipating congestion. Tetramethylpyrazine (TMP), the main active ingredient of *Ligusticum chuanxiong*, has significant antioxidant, anti-inflammatory, and vascular protective effects. However, the protective properties and underlying mechanisms of TMP against endothelial injury-induced insufficient angiogenesis and thrombosis have not been elucidated. Therefore, we aimed to explore the protective effects of TMP on endothelial injury and its antithrombotic effects and study the mechanism. In vitro experiments showed that TMP could alleviate hydrogen peroxide- (H₂O₂-) induced endothelial injury of human umbilical vein endothelial cells (HUVECs) and the protective mechanism might be related to the regulation of MAPK signaling pathway, and its antioxidative and antiapoptotic effects. In vivo experiments showed that TMP restored PTK787-induced damage to intersegmental vessels (ISVs) in Tg(fli-1: EGFP)y1 transgenic (Flik) zebrafish larvae. Similarly, adrenalin hydrochloride- (AH-) induced reactive oxygen species (ROS) production and thrombosis in AB strain zebrafish were inhibited by TMP. RT-qPCR assay proved that TMP could inhibit the expression of fga, fgb, fgg, f7, and von Willebrand factor (vWF) mRNA to exert an antithrombotic effect. Our findings suggest that TMP can contribute to endothelial injury protection and antithrombosis by modulating MAPK signaling and attenuating oxidative stress and antiapoptosis.”

Zhang, Y.,et al. (2022). Tetramethylpyrazine Protects Endothelial Injury and Antithrombosis via Antioxidant and Antiapoptosis in HUVECs and Zebrafish.

Objectives: To study the effects and mechanisms of tetramethylpyrazine (TMP) on tumor necrosis factor- α (TNF- α)-induced inflammatory injury in human coronary artery endothelial cells (HCAEC). **Methods:** HCAEC were randomly divided into four groups: the control group (no treatment), the model group (treated with TNF- α , 50 ng/mL for 24 hours), the TMP group (pre-treated with TMP, 80 μ g/mL for 12 hours followed by TNF- α treatment for 24 hours), and the SIRT1 inhibitor group (pre-treated with TMP and the specific SIRT1 inhibitor EX527 for 12 hours followed by TNF- α treatment for 24 hours). Cell viability was assessed using the CCK-8 method, lactate dehydrogenase (LDH) activity was measured using an LDH assay kit, reactive oxygen species (ROS) levels were observed using DCFH-DA staining, expression of pyroptosis-related proteins was detected by Western blot, and SIRT1 expression was analyzed using immunofluorescence staining. **Results:** Compared to the control group, the model group showed decreased cell viability, increased LDH activity, ROS level and expression of pyroptosis-related proteins, and decreased SIRT1 expression ($P < 0.05$). Compared to the model group, the TMP group exhibited increased cell viability, decreased LDH activity, ROS level and expression of pyroptosis-related proteins, and

increased SIRT1 expression ($P < 0.05$). In comparison to the TMP group, the SIRT1 inhibitor group showed decreased cell viability, increased LDH activity, ROS level and expression of pyroptosis-related proteins, and decreased SIRT1 expression ($P < 0.05$). Conclusions: TMP may attenuate TNF- α -induced inflammatory injury in HCAEC, which is associated with the inhibition of pyroptosis and activation of the SIRT1 signaling pathway. As taken from Chen L-P et. Al. 2024. Zhongguo Dang Dai Er Ke Za Zhi.

Herein, influences of TMP on β -glycerophosphate (β -GP)-stimulated VSMCs and OPG-/- mice were explored. Mouse Aortic Vascular Smooth Muscle (MOVAS-1) cells were stimulated by β -GP with si-caspase-3, si-Gasdermin E (GSDME) or TMP. Increased calcification, reactive oxygen species (ROS) level, Interleukin-1 β (IL-1 β) and Interleukin-18 (IL-18) levels, lactate dehydrogenase (LDH) release, enhanced apoptosis, and activated cysteine-aspartic acid protease-3 (caspase-3)/GSDME signaling were observed in β -GP-stimulated MOVAS-1 cells, which was sharply alleviated by si-caspase-3, si-GSDME or TMP. Furthermore, the impact of TMP on the β -GP-induced calcification and injury in MOVAS-1 cells was abolished by raptinal, an activator of caspase-3. Subsequently, OPG-/- mice were dosed with TMP or TMP combined with raptinal. Calcium deposition, increased nodules, elevated IL-1 β and IL-18 levels, upregulated CASP3 and actin alpha 2, smooth muscle (ACTA2), and activated caspase-3/GSDME signaling in OPG-/- mice were markedly alleviated by TMP, which were notably reversed by the co-administration of raptinal. Collectively, TMP mitigated CAC by inhibiting caspase-3/GSDME mediated pyroptosis. As taken from Yang H et. Al. 2024. Biosci Trends.

Tetramethylpyrazine (TMP) belongs to the active ingredients of the traditional Chinese medicine Chuanxiong, which has a certain protective effect in myocardial ischemia-reperfusion (I/R) injury. It can improve postoperative cardiac function and alleviate ventricular remodeling in acute myocardial infarction patients. However, its specific protective mechanism is still unclear. In this study, a certain concentration of TMP was introduced into I/R mice or H9C2 cells after oxygen-glucose deprivation/reoxygenation (OGD/R) treatment to observe the effects of TMP on cardiomyocyte activity, cytotoxicity, apoptosis, autophagy, pyroptosis, and NLRP3 inflammasome activation. The results displayed that TMP intervention could reduce OGD/R and I/R-induced cardiomyocyte apoptosis, accelerate cellular activity and autophagy levels, and ameliorate myocardial tissue necrosis in I/R mice in a dose-dependent manner. Further, TMP prevented the formation of NLRP3 inflammasomes to suppress pyroptosis by increasing the level of cardiomyocyte autophagy after I/R and OGD/R modelling, the introduction of chloroquine to suppress autophagic activity in vivo and in vitro was further analyzed to confirm whether TMP inhibits NLRP3 inflammasome activation and pyroptosis by increasing autophagy, and we found the inhibitory effect of TMP on NLRP3 inflammasomes and its protective effect against myocardial injury were blocked when autophagy was inhibited with chloroquine. In conclusion, this experiment demonstrated that TMP unusually attenuated I/R injury in mice, and this protective effect was achieved by inhibiting the activation of NLRP3 inflammasomes through enhancing autophagic activity. As taken from Wang K et. Al. 2024. Biochem Pharmacol.

Methods: We established a mouse model of lipopolysaccharide (LPS)-induced ALI and performed single cell RNA sequencing (scRNA-seq). Bioinformatic analyses of the immune, epithelial and endothelial cells were then performed to explore the dynamic changes of the lung tissue microenvironment. We also analyzed the effects of TMP on the cell subtypes, differential gene expression and potential regulation of transcriptional factors involved. Immunohistochemistry and enzyme-linked immunosorbent assay were performed to identify the effects of TMP on immune inflammatory response. Results: We found that TMP efficiently protected against LPS-induced acute lung injury. Results of scRNA-seq showed that the cells were divided into seven major cell clusters, including immune cells, fibroblasts, endothelial cells and epithelial cells. Neither dexamethasone (Dex) nor TMP treatment showed any significant protective effects in these clusters. However, TMP treatment in the LPS-induced ALI model significantly increased follicular helper T cells and reduced CD8⁺ naive T cells, Vcan-positive monocytes and Siva-positive NK cells. In addition, TMP treatment increased the number of basal epithelial cells and lymphatic

endothelial cells (LECs), indicating its protective effects on these cell types. Scenic analysis suggested that TMP likely mitigates LPS-induced injury in epithelial and endothelial cells by promoting FOSL1 in basal epithelial cells and JunB in LECs. Conclusions: Our findings suggest that TMP appears to alleviate LPS-induced lung injury by regulating the immune response, promoting epithelial cell survival and boosting the antioxidant potential of endothelial cells. This study highlights the potential therapeutic use of TMP in the management of ALI. As taken from Wu M et. Al. 2025. PeerJ.

Over the past 2 decades, there has been a growing body of evidence demonstrating the positive effects of ligustrazine on CIS in both in vivo and in vitro studies (Zhu et al., 2021). The mechanism of action of ligustrazine involves several beneficial processes, such as activating free radical scavenging (Yang et al., 2017; Zhang et al., 2018a), reducing BBB destruction (Tan et al., 2015; Gong et al., 2019), inhibiting inflammation (Zhou et al., 2019; Chang et al., 2023), maintaining mitochondrial function (Zhang et al., 2018b), preventing apoptosis (Chang et al., 2007), and promoting oligodendrogenesis and gliogenesis (Zhang et al., 2018a). (...) Studies have demonstrated that following an ischemic stroke, there is a likelihood of significant brain edema and the occurrence of large-area infarcts. Additionally, neurons may undergo extensive necrosis and apoptosis, leading to severe damage to the cell structure (Jurcau and Simion, 2021; Fu et al., 2023). This study conducted a statistical analysis of the included studies and found that ligustrazine exhibits a significant improvement in cerebral edema, reduction in cerebral infarction area, and amelioration of neurological damage. Furthermore, the statistical analysis of outcome indicators in the included studies revealed that ligustrazine can effectively enhance inflammatory factor-related indicators (TNF- α , IL-1 β , and IL-6), oxidative stress-related indicators (SOD, NOS, MDA, and NO), apoptosis indicator caspase-3, and BBB permeability-related protein Claudin-5. (...) The findings of this study demonstrate that ligustrazine can effectively modulate TNF - α , IL-6, and IL-1 β levels in animals with ischemic stroke, thereby improving the damage caused by the stroke.(...) Cerebral ischemia reperfusion injury leads to the production of a large amount of oxygen free radicals, which in turn cause peroxidation reactions and changes in the basic characteristics of cell membranes. This process results in the production of MDA in large quantities. SOD, as a main enzyme responsible for scavenging oxygen free radicals, plays a crucial role in protecting cells against oxidative damage (Korenić et al., 2014; Deng et al., 2022). In this study, mice in the ligustrazine intervention group showed a significant decrease in oxidative stress index levels of NO, NOS, and MDA, as well as a significant increase in the antioxidant index SOD level. These findings suggest that ligustrazine effectively regulates the oxidative balance in brain tissue injury, thereby reducing damage caused by oxygen free radicals and protecting nerve cells.(...) Previous research has demonstrated that ligustrazine has the ability to penetrate the BBB and distribute across various brain regions (Tsai and Liang, 2001). However, its biological half-life when taken orally is limited to 0.5–2 h. Utilizing ligustrazine - loaded liposomes (Xia et al., 2016), transdermal administration drugs (Qi et al., 2002), intranasal administration (Feng et al., 2009), and intraocular administration (Mao et al., 2019) can enhance the efficiency of ligustrazine in crossing the BBB and reaching the brain. Moreover, ligustrazine exhibits protective effects against CIS damage by reducing BBB permeability (Tan et al., 2015; Jin et al., 2021), possibly through the inhibition of the JAK/STAT signaling pathway (Gong et al., 2019) and the reduction of MMP-9 levels (Jin et al., 2021). Claudin-5, a specific protein found in the tight junctions of brain microvascular endothelial cells, has been identified as an inducer of BBB formation (Ueno, 2007). The analysis conducted in this study reveals that ligustrazine can reduce the expression of Caspase-3 and increase Claudin-5 levels. These findings suggest that ligustrazine may protect against cerebral ischemic injury by reducing apoptosis and repairing the BBB. As taken from Wang Z et. Al. 2024. Front Pharmacol.

5.6. Carcinogenicity

“To explore the role of hypercoagulation in the metastasis of carcinoma. The effect of Tetramethylpyrazine (TTMP) on platelet functions among the 25 advanced cases of lung carcinoma, and 26 matched control subjects were investigated in the study. Their ages varied from

31-86 years (mean 58.2) in lung carcinoma group (13 male, 12 female) and 36 to 61 (mean 52.9) in the control group (16 male, 10 female). The pathologic types were as follows: 7 cases of squamous cell cancer, 12 adenocarcinoma, 2 small cell carcinoma and 4 undistinguished type. The TNM stage revealed 14 cases in stage IIIa, 3 in stage IIIb and 8 in stage IV. The site of metastasis included mediastinal lymph node, pleura, supraclavicular lymph node, brain, spine, costa, skin and pericardium. The levels of plasma TXB2, 6-keto-PGF1 alpha, VIII:C, vWF, AT-III:a, AT-III:Ag, Fg and blood PAdT, PAgT were measured before and after the intravenous infusion of 80 mg TTMP in patients with lung carcinoma. The levels of TXB2, 6-keto-PGF1 alpha, VIII:C, vWF and Fg in lung carcinoma group were significantly elevated, while the levels of PAdT was greatly decreased, compared with the control group, no significant differences in levels of PAgT, AT-III:a and AT-III:Ag were found between the two groups. After the infusion of TTMP the levels of PAdT, PAgT, VIII:C, dWF and Fg were decreased significantly, while TXB2, 6-keto-PGF1 alpha, AT-III:a and AT-III:Ag remained unchanged. TTMP inhibits the adhesion and aggregatory functions of blood platelet and the activity of coagulation factors. It might be one of the mechanisms of TTMP's antimetastasis of lung carcinoma." As taken from Chen SX et al. *Zhongguo Zhong Xi Yi Jie He Za Zhi*. 1997. 17(9), 531-3. PubMed, 2010

"Cyclooxygenase (COX)-2 plays an important role in tumorigenesis and has been implicated to be a critical factor for invasion and metastasis of lung cancer. Tetramethylpyrazine (TMP), an effective component of the traditional Chinese medicine Chuanxiong, has been traditionally used in treating neurovascular and cardiovascular diseases. Recently TMP has been reported to have beneficial effect in cancer patients. However, the function and the mechanism of TMP in lung cancer have not been elucidated to date. In this study, we investigated the in vitro and in vivo effect of TMP in tumorigenesis and whether COX-2 is a molecular target of TMP. We showed that TMP exhibited a dose- and time-dependent inhibition on A549 cell proliferation by suppressing cell cycle progression. In vitro treatment of A549 cells with TMP resulted in a significant inhibition of invasion, associated with reduced activities of COX-2 and MMP-2/TIMP-2. Furthermore, in vivo experiments showed that TMP significantly suppressed metastatic growth of A549 cells and COX-2 expression in metastatic nude mouse model. This preclinical study provides the first evidence for the novel anti-tumor effects of TMP as a COX-2 pathway inhibitor in human adenocarcinoma cell line A549. These studies suggest that TMP may serve as an effective agent for the treatment and chemoprevention of non-small cell lung cancer." As taken from Zheng CY et al. *Int. J. Oncol*. 2012. 40(6), 2029-37. PubMed, 2013

"Tumor multidrug resistance (MDR) has become the major obstacle to cancer chemotherapy. Recent studies suggest that tetramethylpyrazine (TMP) could reverse tumor MDR although the mechanism by which TMP overcomes tumor MDR remains elusive. Therefore, in this study, we examined the effects of TMP on MDR in drug-resistant breast cancer cells and investigated the underlying mechanisms. MCF-7 cells and the derived P-glycoprotein (Pgp) overexpressing MCF-7/dox cells were treated with TMP, and their growth was examined by MTT assay. Doxorubicin accumulation in the cells was evaluated by flow cytometry, and the expression of Pgp was detected by Western blot and RT-PCR analysis. The results showed that TMP increased the intracellular concentration of doxorubicin and inhibited Pgp-mediated efflux of doxorubicin in a dose-dependent manner. Moreover, TMP inhibited the ATPase activity of P-gp and suppressed the expression of Pgp in MCF-7/dox cells. Taken together, these data suggest that TMP has potential application in the treatment of chemotherapy-resistant breast cancer." As taken from Zhang Y et al. *Med. Oncol*. 2012a. 29(2), 534-8. PubMed, 2013

"Tetramethylpyrazine (TMP) is an effective component of the traditional Chinese medicine Chuanxiong, which has been reported to have beneficial effects in various types of cancer. However, the activity and mechanism of action of TMP in osteosarcoma (OS) have not been elucidated to date. The aim of the present study was to investigate the inhibitory effect of TMP on OS and its underlying mechanism of action. OS cells were treated with various concentrations of TMP for 48 h. BALB/c nude mice with OS were treated with an intraperitoneal injection of TMP at a dose of 100 mg/kg every other day for 28 days. Cell proliferation was evaluated using an MTT

assay. Cell cycle and apoptosis were measured using flow cytometry. The protein expression of nuclear and cytosolic nuclear factor- κ B (NF- κ B) p65, BCL-2 and cyclin D1 was measured using western blot analysis. TMP inhibited the proliferation of OS cells (MG-63, SAOS-2 and U2OS) in a dose-dependent manner. Additionally, TMP significantly induced apoptosis and G0/G1 arrest in MG-63 OS cells ($P < 0.05$). TMP upregulated the protein expression of cytosolic NF- κ B p65, while downregulating the protein expression of nuclear NF- κ B p65, BCL-2 and cyclin D1. Furthermore, TMP exerted a significant antitumor effect against OS in a xenograft tumor mouse model and exhibited a low toxicity. The present study provided fundamental evidence for the application of TMP in chemotherapy against OS." As taken from Wang Y et al. 2013. *Mol. Med. Rep.* 8(4), 984–8. PubMed, 2013

"Tetramethylpyrazine (TMPZ) is one of the active compounds extracted from the traditional Chinese medicinal herb Chuanxiong and several studies have shown it to possess anticancer properties. However, its effectiveness in gastric cancer and its cellular mechanisms are relatively unknown. The present study aimed to investigate the effect of TMPZ on SGC7901 cells, and it was demonstrated that a high dose of TMPZ inhibited cell viability and induced apoptosis by stimulating AMP-activated protein kinase (AMPK) through the generation of reactive oxygen species (ROS). Furthermore, TMPZ-induced apoptosis resulted in the sequential events beginning with the translocation of Bax, the collapse of mitochondrial membrane potential ($\Delta\Psi_m$), the release of cytochrome c and the activation of caspase-9 and -3. Each of these events was inhibited by compound C, a pharmacological inhibitor of AMPK. To the best of our knowledge, these results demonstrate for the first time that the induction of apoptosis by TMPZ in gastric cancer cells is associated with the activation of the ROS/AMPK pathway. AMPK activation induces apoptosis through the mitochondrial apoptotic pathway. In addition, these results raise the possibility that TMPZ may have a future therapeutic role in gastric cancer." As taken from Yi B et al. 2013. *Oncol. Lett.* 6(2), 583–589. PubMed, 2013

"The survival in patients with malignant gliomas still remains limited and novel treatment strategies are urgently needed. Tetramethylpyrazine (TMP) extracted from the Chinese herb Chuanxiong, has been suggested to have a therapeutic potential towards glioma primarily through its neural protection activity. However, the exact mechanisms correlating TMP's antitumor function and neural protection have not been yet elucidated. Thus, this study aimed to investigate TMP's molecular target in tumor inhibition and neural protection. The primary cultured cerebral neurocytes were treated with 100 μ M TMP for 14 days in vitro. We found TMP can effectively promote neurons survival, compared to controls. TMP effectively inhibits H₂O₂-induced rise of $[Ca^{2+}]_i$ and glutamate releasing in cerebral neurocytes, compared to controls. In addition, we verify previous results that TMP significantly decreases the migration and proliferation of C6 glioma cells. Using glioma-neuronal co-culturing system, we further confirm TMP bioactivity in inhibition of glioma cells and protection of cerebral neurocytes. More importantly, our study demonstrates that the expression of chemokine receptor, CXCR4, which plays a key role in tumor development and various neurodegenerative diseases, is significantly decreased in both cerebral neurocytes and C6 glioma cells with TMP treatment, cultured alone or co-cultured. Compared with CXCR4 antagonist, AMD3100, TMP is more effective on glioma inhibition and neural protection. Glutamate concentration in medium of co-culturing system was lower after treatment with 100 μ M TMP. Therefore, our findings suggest that TMP-mediated suppression of C6 gliomas and neural protection involves inhibition of CXCR4 expression. Thus, this study provides new insights into TMP's therapeutic potential in the treatment of malignant gliomas." As taken from Chen Z et al. 2013. *Cancer Lett.* 336(2), 281–9. PubMed, 2013

"2,3,5,6-Tetramethylpyrazine (TMP) is known as a composition of Ephedra sinica and it has been used in the treatment of several disorders such as asthma, heart failure, rhinitis, and urinary incontinence. It has been reported that TMP inhibits melanoma metastasis and suppression angiogenesis by VEGF. OBJECTIVE: The inhibitory activity of melanogenic proteins by TMP was confirmed in UVA-induced melanoma/keratinocyte co-culture system in this paper. METHODS: The melanin content, cell viability and cytokines release such as TNF α , IL-1 β , IL-8 and GM-CSF were

measured by ELISA assay. In addition, TRP1, MITF and MAPK signaling protein expression were also evaluated by Western blotting analysis. RESULTS: Decreasing melanogenic factors (TRP1, MITF, and MAPK) and factors (TNF α , IL-1 β , IL-8, and GM-CSF) improving skin cancer and inflammation were identified. CONCLUSION: It suggests that TMP can serve as a potent candidate for regulation of melanogenesis". As taken from Yeom GG et al. 2014. *Int. Immunopharmacol.* 18(2), 262-9. PubMed, 2015

"Hepatocellular carcinoma (HCC) is one of the most common types of liver cancers with high recurrence rate and mortality rate. Recent studies have indicated that tetramethylpyrazine (TMP), a purified chemical extracted from *Ligusticum wallichii* Franchet (ChuanXiong), possessed antitumor effects on HCC, but detailed mechanism remains unclear. Our study aims at investigating the antitumor effect of TMP on HCC and its underlying mechanism. We found that TMP inhibited cell proliferation of HepG2 cells in a dose-dependent way, and xenograft tumor models also indicated that high concentrations of TMP administration inhibited tumor growth. Next, flow cytometric analysis and transmission electron microscope images showed that TMP enhanced cell apoptosis in HepG2 cells, and western blot results showed that TMP promoted cleavage of caspase-3 and PARP in vitro and in vivo. We also found that TMP caused autophagy in HCC in vitro and in vivo. In order to examine the role of autophagy in TMP-induced apoptosis, 3-methyladenine (3-MA) was used to block the action of autophagy. Our data showed TMP-induced autophagy might be a pro-apoptosis process in HCC. Furthermore, the results of anti-oxidative enzymes and oxidation-sensitive fluorescent probe 2, 7-dichlorofluorescein diacetate (DCFH-DA) indicated that TMP induced ROS generation and inhibition of ROS diminished the anticancer function of TMP. In conclusion, our studies provide new insights into the mechanisms underlying the antitumor effect of TMP and suggest that TMP can be a novel therapeutic regimen for HCC." As taken from Cao J et al. 2015a. *Int. Immunopharmacol.* 26(1), 212-20. PubMed, 2016

"Hepatocellular carcinoma (HCC) is the fifth most common cancer. An important approach to control HCC is chemoprevention. This study aims at investigating the antitumor effect of Tetramethylpyrazine (TMP). Rats were injected with N-Nitrosodiethylamine (DEN) to establish HCC. Tumor development was observed. Liver function was evaluated. Apoptosis and cell cycle arrest-related makers and signaling cascades were determined by Western blot, RT-PCR and flow cytometric analysis. The administration of TMP could significantly inhibit tumor development in DEN-induced HCC rats, shown by reduced incidence of tumor, decreased number of tumor nodules and reduced maximal size of tumor. DEN-induced increase of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase and alkaline phosphatase activities were significantly inhibited by TMP. TMP exhibited inhibitory effect on HCC through induction of apoptosis and cell cycle arrest in rats. TMP induced apoptosis through increasing Bax, decreasing Bcl-2, increasing the release of cytochrome c, and activating caspase, which consisted of the mitochondrial apoptotic pathway. TMP induced G2/M cell cycle arrest through down-regulation of cyclin B1/cdc2. In addition, inhibition of Akt and ERK signaling and the antioxidant activities of TMP may also contribute to its antitumor effect. These data provide new insight into the mechanisms underlying the antitumor effect of TMP." As taken from Cao J et al. 2015b. *J. Biol. Regul. Homeost. Agents* 29(2), 297-306. PubMed, 2016

"OBJECTIVE: To further explore the function of combine use of tetramethylpyrazine (TMP) and cisplatin (DDP) in lung carcinoma. METHODS: We used the combination drug to treat Lewis lung cancer mice, investigated the expression level of vascular endothelial growth factor (VEGF), Kruppel-like factor 4 (KLF4) and A disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1) and to further explore the inhibitory effects and potential mechanism of TMP combined with DDP on tumor angiogenesis. RESULTS: The tumor growth was suppressed in TMP group, DDP group and TMP combined with DDP group. Furthermore, the weights and volume of tumor, the expression level of VEGF, KLF4 and ADAMTS1 were found significantly changed between experiment group and control group. These findings suggest that TMP with DDP had additional or synergistic effects to inhibit the tumor growth effectively, might be achieved through reducing the expression of angiogenesis promoting factor VEGF and increasing expression of angiogenesis

inhibitors KLF4 and ADAMTS1. CONCLUSION: KLF4 and ADAMTS1 may be synergically involved in the angiogenesis in mouse Lewis lung cancer through the different signal ways.” As taken from Tang JH et al. 2017. Asian Pac. J. Trop. Med. 10(8), 813-818. PubMed, 2018

2,3,5,6-Tetramethylpyrazine is suspected to be a carcinogen. The CAESAR Carcinogenicity model in the VEGA (Q)SAR platform predicts that the chemical is a Carcinogen (moderate reliability). As taken from ECHA, 2016.

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.

ECHA Annex III has been withdrawn as of April 2023: “The list of substances that are unlikely to benefit from the reduced information requirements, the so-called ‘Annex III inventory’, has been withdrawn.”

Ligusticum chuanxiong Hort (known as Chuanxiong in China, CX) is one of the most widely used and long-standing medicinal herbs in China. Tetramethylpyrazine (TMP) is an alkaloid and one of the active components of CX. Over the past few decades, TMP has been proven to possess several pharmacological properties. It has been used to treat a variety of diseases with excellent therapeutic effects. Here, the pharmacological characteristics and molecular mechanism of TMP in recent years are reviewed, with an emphasis on the signal-regulation mechanism of TMP. This review shows that TMP has many physiological functions, including anti-oxidant, anti-inflammatory, and anti-apoptosis properties; autophagy regulation; vasodilation; angiogenesis regulation; mitochondrial damage suppression; endothelial protection; reduction of proliferation and migration of vascular smooth muscle cells; and neuroprotection. At present, TMP is used in treating cardiovascular, nervous, and digestive system conditions, cancer, and other conditions and has achieved good curative effects. The therapeutic mechanism of TMP involves multiple targets, multiple pathways, and bidirectional regulation. TMP is, thus, a promising drug with great research potential.

Lin, J., et al. (2022). Tetramethylpyrazine: A review on its mechanisms and functions. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie, 150, 113005.

5.7. Irritation/immunotoxicity

“2,3,5,6-Tetramethylpyrazine (TMP) is known as a composition of Ephedra sinica and it has been used in the treatment of several disorders such as asthma, heart failure, rhinitis, and urinary incontinence. It has been reported that TMP inhibits melanoma metastasis and suppression angiogenesis by VEGF. OBJECTIVE: The inhibitory activity of melanogenic proteins by TMP was confirmed in UVA-induced melanoma/keratinocyte co-culture system in this paper. METHODS: The melanin content, cell viability and cytokines release such as TNF α , IL-1 β , IL-8 and GM-CSF were measured by ELISA assay. In addition, TRP1, MITF and MAPK signaling protein expression were also evaluated by Western blotting analysis. RESULTS: Decreasing melanogenic factors (TRP1, MITF, and MAPK) and factors (TNF α , IL-1 β , IL-8, and GM-CSF) improving skin cancer and inflammation were identified. CONCLUSION: It suggests that TMP can serve as a potent candidate for regulation of melanogenesis”. As taken from Yeom GG et al. 2014. Int. Immunopharmacol. 18(2), 262-9. PubMed, 2015

“OBJECTIVE: To observe the effect of Tetramethyl pyrazine (TMP) on the cytokines and inflammatory mediators in the serum and the synovial fluid of collagen-induced arthritis (CIA) rats, and further to investigate its possible mechanisms for treating rheumatoid arthritis (RA). METHODS: Type II CIA rat model was established. Rats in the TMP group were administered with TMP at 50 mg/kg and 100 mg/kg, once daily. Dexamethasone at 2.0 mg/kg was intramuscularly

injected to those in the Dexamethasone treated group, once daily. Normal saline at 2 mL/kg was given to those in the normal control group and the model group, once daily. All medication was started from the 7th day, lasting to the 35th day. CIA rats' foot swelling degree was observed. Contents of serum IL-1, IL-6, IL-2, NO and PGE₂ in the synovial fluid were detected by radioimmunoassay and nitrate reduction method. RESULTS: Compared with the normal group, the foot swelling obviously increased, contents of NO and PGE₂ in the synovial fluid were obviously elevated in the model group ($P<0.01$). Compared with the model group, the foot swelling could be obviously inhibited by 100 mg/kg TMP and Dexamethasone; serum levels of IL-1 and IL-6 obviously decreased, serum IL-2 level obviously increased, contents of NO and PGE, decreased ($P<0.01$). TMP 50 mg/kg could obviously inhibit the foot swelling of CIA rats ($P<0.01$). There was no statistical difference in other indices ($P>0.05$). CONCLUSIONS: TMP at 100 mg/kg showed obvious inhibition on CIA rats. Its inhibitory effect might be correlated to inhibiting activities of endogenous cytokines and the generation of inflammatory mediators in inflammation local regions, improving contents of anti-inflammation cytokines, and inducing the balance of the inflammatory cytokine network." As taken from Mu CX et al. 2014. Zhongguo Zhong Xi Yi Jie He Za Zhi 34(2), 214-7. PubMed, 2015

"OBJECTIVE: To explore the effect of combination therapy of tetramethylpyrazine (TMP) with methotrexate (MTX) on collagen induced arthritis (CIA) rats. METHODS: Totally 55 male SD rats were stratified by body weight. Nine of them were randomly recruited as the normal control group. The rest 46 were immunized with type II bovine collagen (C II) for establishing rheumatoid arthritis (RA) model. Forty successfully modeled rats were randomly divided into 4 groups according to swollen toe degree, i.e., the CIA group, the TMP group, the MTX group, and the TMP plus MTX group, 10 in each group. Rats in the MTX group were administered with MTX (1.2 mg/kg), once per week for 4 continuous weeks. Those in the TMP group were administered with 40 mg/kg TMP, once per day for 10 continuous days, and then discontinued for 7 successive days, and continued for another 10 successive days. Rats in the TMP plus MTX group were administered with a mixture of equal dose MTX and TMP, and when MTX was discontinued, TMP was administered according to the way in the TMP group. Equal volume of saline solution was given to rats in the normal control group and the CIA group. Clinical parameters including ankle width (mediolateral diameter) and hindpaw swelling were measured at day 0, 4, 11, 18, and 26 after treatment. Rats were sacrificed 28 days after treatment, their knee joints and ankle joints were collected for pathological analyses. Serum levels of IL-1 β , IL-6, and IL-17A were detected by ELISA. Changes of fibrinogen (FIB) and platelet aggregation rate (PAg) were detected. RESULTS: Compared with the normal control group, the ankle width and hindpaw swelling increased significantly ($P<0.01$), contents of FIB and PAg increased obviously ($P<0.05$, $P<0.01$), serum levels of IL-1 β , IL-6, and IL-17 increased remarkably ($P<0.01$) in the CIA group. Obvious cell proliferation, inflammatory cell infiltration, hyperemia and edema of synovial tissues could be seen. Pannus formed and immersed in cartilages, resulting in necrosis. Compared with the model group, changes of ankle width and hindpaw swelling were all alleviated in each medicated group ($P<0.05$, $P<0.01$). Of them, the effect was superior in the MTX group to that of the TMP group and the MTX plus TMP group ($P<0.05$, $P<0.01$). Contents of FIB, serum levels of IL-1 β and IL-6 decreased significantly in the MTX group ($P<0.05$). Contents of FIB, serum levels of IL-1 β and IL-6 decreased significantly in the TMP group and the MTX plus TMP group ($P<0.05$). Besides, serum levels of FIB and IL-6 were obviously lower in the MTX plus TMP group than in the TMP group and the MTX group ($P<0.01$). Levels of PAg and IL-17A were more significantly lowered in the TMP group than in the MTX plus TMP group and the MTX group. Pathological changes could be alleviated in each medicated group, with the optimal effect obtained in the MTX plus TMP group. CONCLUSION: Combination of TMP with MTX could significantly ameliorate inflammatory reactions and FIB contents of CIA rats." As taken from Bian YQ et al. 2015. Zhongguo Zhong Xi Yi Jie He Za Zhi 35(6), 724-9. PubMed, 2016

"Experimental studies have demonstrated the beneficial effects of tetramethylpyrazine (TMP) against ischemic stroke and highlighted its crucial role in anti-inflammatory activity. This study provides evidence of an alternative target for TMP and sheds light on the mechanism of its anti-

inflammatory action against ischemic brain injury. We report a global inhibitory effect of TMP on inflammatory cell intracerebral activation and infiltration in a rat model of permanent cerebral ischemia. The results of immunohistochemistry, enzymatic assay, flow cytometric analysis, and cytological analysis revealed that intraperitoneal TMP administration reduced neuronal loss, macrophage/microglia activation, brain parenchyma infiltrative neutrophils, and circulating neutrophils after cerebral ischemia. Biochemical studies of cultured neutrophils further demonstrated that TMP attenuated neutrophil migration, endothelium adhesion, spontaneous nitric oxide (NO) production, and stimuli-activated NO production after cerebral ischemia. In parallel with these anti-neutrophil phenomena, TMP also attenuated the activities of ischemia-induced inflammation-associated signaling molecules, including plasma high-mobility group box-1 protein (HMGB1) and neutrophil toll-like receptor-4 (TLR4), Akt, extracellular signal-regulated kinase (ERK), and inducible nitric oxide synthase. Another finding in this study was that the anti-neutrophil effect of TMP was accompanied by a further elevated expression of NF-E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in neutrophils after cerebral ischemia. Taken together, our results suggest that both the promotion of endogenous anti-inflammatory defense capacity and the attenuation of pro-inflammatory responses via targeting of circulating neutrophils by elevating Nrf2/HO-1 expression and inhibiting HMGB1/TLR4, Akt, and ERK signaling might actively contribute to TMP-mediated neuroprotection against cerebral ischemia." As taken from Chang CY et al. 2015. *Biochem. Biophys. Res. Commun.* 463(3), 421-7. PubMed, 2016

"CONTEXT: Inflammation after spinal cord injury (SCI) may be responsible for further neural damages and therefore inhibition of inflammatory processes may exert a neuroprotection effect. OBJECTIVES: To assess the efficacy of some non-conventional herbal medications including sulforaphane, tanshinone IIA, and tetramethylpyrazine in reducing inflammation and compare them with a known effective anti-inflammatory agent (interleukin-10 (IL-10)). METHODS: We searched relevant articles in Ovid database, Medline (PubMed) EMBASE, Google Scholar, Cochrane, and Scopus up to June 2013. The efficacy of each treatment and study powers were compared using random effects model of meta-analysis. To our knowledge, no conflict of interest exists. RESULTS: Eighteen articles entered into the study. The meta-analysis revealed that exogenous IL-10 was more effective in comparison with the mentioned herbal extracts. The proposed pathways for each medication's effect on reducing the inflammation process are complex and many overlaps may exist. CONCLUSION: IL-10 has a strong effect in the induction of neuroprotection and neurorecovery after SCI by multiple pathways. Tetramethylpyrazine has an acceptable influence in reducing inflammation through the up-regulation of IL-10. Outcomes of sulforaphane and tanshinone IIA administration are acceptable but still weaker than IL-10" As taken from Koushki D et al. 2015. *J. Spinal Cord Med.* 38(1), 13-22. PubMed, 2016

"Objective To investigate the effects of tetramethylpyrazine (TMP) on experimental autoimmune myasthenia gravis (EAMG) in rats and explore the possible immune regulation mechanism. Methods Lewis rats were randomly divided into 4 groups: control group, EAMG group, TMP low-dose group (TMP-L, 10 mg/kg) and TMP high-dose group (TMP-H, 20 mg/kg). Except the control group, the other 3 groups were subjected to EAMG modeling. The body mass was determined and the symptoms of muscular weakness in rats were scored by Lennon EAMG criteria. The expressions of acetylcholine receptor (AChR), IgG and C3 in neuromuscular junctions were examined by immunofluorescence. The levels of R97-116 antibody (IgG1, IgG2a, IgG2b), tumor necrosis factor α (TNF- α), interferon γ (IFN- γ) and interleukin 10 (IL-10) were detected by ELISA. Results TMP decreased the scores in the evaluation of the symptoms of muscle weakness in EAMG rats. Moreover, TMP administration significantly inhibited AChR expression and increased IgG and C3 expression in neuromuscular junctions of EAMG rats. In addition, after TMP treatment, the levels of IgG1, IgG2a and TNF- α were remarkably reduced, while IL-10 level was elevated. However, there was no significant change in serum levels of IgG2b and IFN- γ . Conclusion TMP can exert inhibitory effects on Th1, Th2 and B cells and modulate the levels of IgG1, IgG2a, TNF- α and IL-10. All these effects contribute to relieve the symptoms of muscle weakness in EAMG rats." As

taken from Ren Y et al. 2016. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 32(11), 1471-1474. PubMed, 2018

“OBJECTIVE: To investigate the effects of ligustrazine (LTZ) on airway inflammation in a mouse model of neutrophilic asthma (NA). METHODS: Forty healthy C57BL/6 female mice were randomly divided into 4 groups using a random number table, including the normal control, NA, LTZ and dexamethasone (DXM) groups, with 10 rats in each group. The NA mice model was established by the method of ovalbumin combined with lipopolysaccharide sensitization. At 0.5 h before each challenge, LTZ and DXM groups were intraperitoneally injected with LTZ (80 mg/kg) or DXM (0.5 mg/kg) for 14 d, respectively, while the other two groups were given the equal volume of normal saline. After last challenge for 24 h, the aerosol inhalation of methacholine was performed and the airway reactivity was measured. The bronchoalveolar lavage fluid (BALF) was collected. The Wright-Giemsa staining was used for total white blood cells and differential counts. The levels of cytokines interleukin (IL)-17 and IL-10 were detected by enzyme-linked immunosorbent assay. The pathological change of lung tissue was observed by hematoxylin eosin staining. RESULTS: The airway responsiveness of the NA group was significantly higher than the normal control group ($P<0.05$), while those in the LTZ and DXM groups were significantly lower than the NA group ($P<0.05$). The neutrophil and eosinophil counts in the LTZ and DXM groups were significantly lower than the NA group ($P<0.05$), and those in the LTZ group were significantly lower than the DXM group ($P<0.05$). There were a large number of peribronchiolar and perivascular inflammatory cells in infiltration in the NA group. The airway inflammation in the LTZ and DXM groups were significantly alleviated than the NA group. The infiltration in the LTZ group was significantly reduced than the DXM group. Compared with the normal control group, the IL-17 level in BALF was significantly increased and the IL-10 level in BALF was significantly decreased in the NA group ($P<0.05$). LTZ and DXM treatment significantly decreased IL-17 levels and increased IL-10 levels compared with the NA group ($P<0.05$), and the changes in the above indices were more significant in the LTZ group ($P<0.05$). CONCLUSION: LTZ could alleviate the airway inflammation in the NA mice model through increasing the IL-10 level and decreasing the IL-17 level.” As taken from Liu XM et al. 2018. Chin. J. Integr. Med. 24(5), 353-358. PubMed, 2019

“Asthma is an inflammatory disease which affects millions of people worldwide. Therefore, it is necessary to search for new sources of therapies for the treatment of these patients in order to improve their quality of life. From content analysis of literature of new therapeutic targets, there are various targets and drugs reported to be promising for the treatment of asthma. Interleukins involved in inflammatory processes are often presented as candidate targets for new drugs. The action of such therapeutics would not only affect interleukins, but also their receptors. Small molecules (e.g. ligustrazine and SP600125) and large molecule antibodies (e.g. lebrikizumab, benralizumab, dupilumab) are being considered as novel agents for the pharmacotherapy of asthma. Therefore, through this research, we can see advances in the search for new targets and promising drugs to treat asthma. It is expected that these new drug candidates will eventually be approved and marketed so that asthma patients can use them to enhance their quality of life.” As taken from Alves MF et al. 2018. Mini Rev. Med. Chem. 18(8), 684-696. PubMed, 2019

Ligusticum chuanxiong Hort (Ligusticum; Apiaceae) (accepted name, Ligusticum striatum DC, on „The Plant List” for the latest version) is a Chinese herbal medicine (CHM) which mainly distributed in Sichuan Basin, China. Chuanxiong is the dried rhizome of Ligusticum chuanxiong Hort. Ligustrazine, also known as tetramethylpyrazine (TMP), is a main active fraction of chuanxiong. The aim of this study was to clarify the underlying mechanisms by which TMP protect against psoriasis-like inflammation in keratinocytes. Here, we demonstrated that TMP alleviated the severity and PASI scores of IMQ-induced psoriasis-like skin lesion in vivo. For the histopathology level, TMP inhibited the over-proliferation of keratinocytes in the epidermis and the substantial immune cells influx in dermis. For the mechanism of the ability of TMP on regulating inflammation, we confirmed that TMP regulate the TRAF6/c-JUN/NFκB signaling pathway through analyzing the proteomics profiling and verifying the expression of TRAF6, pho-c-Jun, pho-NFκB, so that the downstream psoriasis-relevant genes transcribed by c-JUN or NFκB were down-regulated.

Furthermore, we predicted TRAF6 as the potential binding point of TMP. Accordingly, our study demonstrated that TMP regulated psoriasis-like inflammation through inhibiting TRAF6/c-JUN/NFκB signaling pathway in keratinocytes, which potentially provides evidence of the mechanism of TMP in the treatment and prevention of psoriasis.

Jiang, R., et al. (2022). Ligustrazine alleviates psoriasis-like inflammation through inhibiting TRAF6/c-JUN/NFκB signaling pathway in keratinocyte.

5.8. All other relevant types of toxicity

“The metabolic character of tetramethylpyrazine (TMPz) in rat liver microsomes was studied in vitro and in vivo to identify which isoforms of cytochrome P450 were responsible for TMPz metabolism in rats. METHOD: Set up UV- HPLC method of TMPz, determine concentration of TMPz and its formation in rat plasma and liver microsomes incubation solution, analyze the correlation between TMPz's metabolic eliminate rate and each inducer. CONCLUSION: Results suggest that CYP3A plays a major role in TMPz metabolism in rats.” As taken from Zhongguo Zhong Yao Za Zhi. 2006. 31(23), 1971-5.

“A study done with rats evaluate the role of microcirculatory disorder and the therapeutic effectiveness of tetramethylpyrazine (TMP) on intestinal mucosa injury in rats with acute necrotizing pancreatitis (ANP). A total of 192 Sprague-Dawley rats were randomly divided into three groups: normal control group (C group), ANP group not treated with TMP (P group), ANP group treated with TMP (T group). An ANP model was induced by injection of 50 g/L sodium taurocholate under the pancreatic membrane (4 mL/kg). C group received isovolumetric injection of 9 g/L physiological saline solution using the same method. T group received injection of TMP (10 mL/kg) via portal vein. Radioactive biomicrosphere technique was used to measure the blood flow at 0.5, 2, 6 and 12 h after the induction of ANP. Samples of pancreas, distal ileum were collected to observe pathological changes using a validated histology score. Intestinal tissues were also used for examination of myeloperoxidase (MPO) expressed intracellularly in azurophilic granules of neutrophils. The blood flow was significantly lower in P group than in C group ($P<0.01$). The pathological changes were aggravated significantly in P group. The longer the time, the severer the pathological changes. The intestinal MPO activities were significantly higher in P group than in C group ($P<0.01$). The blood flow of intestine was significantly higher in T group than in P group after 2 h ($P<0.01$). The pathological changes were alleviated significantly in T group. MPO activities were significantly lower in T group than in P group ($P<0.01$ or $P<0.05$). There was a negative correlation between intestinal blood flow and MPO activity ($r = -0.981$, $P<0.01$) as well as between intestinal blood flow and pathologic scores ($r = -0.922$, $P<0.05$). In conclusion, TMP attenuates MPO activity and improves intestinal microcirculation. Its protective effects against intestinal injury may be attributed to improving microcirculation and further preventing accumulation of neutrophils. Early use of TMP seems to be effective in ameliorating intestinal injury.” (Zhang et al., 2006)

“Ligustrazine, a bioactive component contained in Chuanxiong (*Ligusticum chuanxiong* Hort), is widely applied in the treatment of vascular diseases in China, e.g. myocardial and cerebral infarction. The aim of this study was to perform a meta-analysis of randomized controlled trials (RCTs) to evaluate the clinical effect of Ligustrazine on diabetic nephropathy (DN). PUBMED, EMBASE, the Cochrane Central Register of Controlled Trials (CENTRAL), Chinese Biological Medicine Database (CBM), China National Knowledge Infrastructure (CNKI) Database, etc. were searched by computer and manual methods to identify RCTs that were used to evaluate the clinical effect of Ligustrazine on DN patients. Twenty five studies comprising 25 RCTs were involved including 1645 patients (858 in the treatment group and 787 in the control group). The meta-analysis suggests that compared with the control group, Ligustrazine injection has a significant therapeutic effect on improving renal function (blood urea nitrogen [BUN] and serum creatinine [SCr]) and reducing in urine protein (24 h urine protein, urine micro albumin and urinary albumin excretion rate [UAER]) in DN patients.” As taken from Wang B et al. Am. J. Chin. Med. 2012. 40(1), 25-37. PubMed 2013

“Tetramethylpyrazine (TMP) and butylidenephthalide (BP) are two bioactive components isolated from *Ligusticum chuanxiong* Hort and *Angelica sinensis*, respectively. These two traditional Chinese medicines have been widely used in clinical treatments for vascular disease. The mechanism by which TMP and BP protect endothelial cells (ECs) against oxidative stress remains unknown, as does their effects on the steady state of the lipidome of ECs. Here, we demonstrate that both compounds protect EA.hy926 cells against H₂O₂ induced injury in a dose-dependent manner. We then apply an integrated analysis of the lipidome and signal transduction pathways to explore the underlying mechanism of their protective effects. We found that TMP elevates the content of several phosphatidylcholine (PC) species, reduces the release of arachidonic acid (AA) and inhibits the phosphorylation of cytosolic phospholipase A₂ (cPLA₂). Compared to eicosatetraenoic acid (ETYA), a cPLA₂ inhibitor, TMP preferentially increases the content of arachidonoyl PCs. We also show that BP mainly elevates the pool of phosphatidylinositol (PI) species and inhibits the phosphorylation of both phospholipase C(γ) (PLC(γ)) and extracellular signal-regulated kinase 1/2 (ERK1/2). In contrast, specific inhibition of ERK1/2 by PD98059 decreases the cell viability and increases pool of phosphatidylserine (PS). Taken together, these results demonstrate that TMP protects oxidatively stressed ECs through inhibition of cPLA₂ and preferential increase of arachidonoyl PC levels. Conversely, the effects of BP are tied to inhibition of PLC(γ) and an increase in PI levels. The current work suggests that the interaction of the lipidome and phospholipases can serve as a promising therapeutic target in oxidatively stressed ECs.” As taken from Yang J et al. *Mol. Biosyst.* 2012. 8(6), 1789-97. PubMed, 2013

“The pregnane X receptor (PXR) is a key regulator of CYP3A4, which is involved in catalyzing the metabolic conversion of a number of endogenous substrates. In this study, we screened 22 compounds isolated from traditional Chinese herbal medicines using luciferase reporter gene assays for inspecting their capabilities in inducing PXR-mediated transactivation of CYP3A4 expression. In addition, the mRNA and protein expressions of CYP3A4 and PXR as well as the enzymatic activities of CYP3A4 were analyzed by real-time PCR, Western blot analysis and UPLC-MS/MS-based metabolite assay in LS174T cells. Huperzine A, ligustrazine and oridonin were identified to be the inducers of CYP3A4. These compounds induced the CYP3A4 reporter luciferase activity, and up-regulated CYP3A4 mRNA and protein levels significantly. Besides, huperzine A, ligustrazine and oridonin significantly up-regulated enzymatic activities of CYP3A4. However, the three compounds showed no effects on PXR mRNA and protein expression. To our knowledge, it is the first identification of these three compounds as PXR activators to induce CYP3A4. These results indicate that huperzine A, ligustrazine and oridonin induced CYP3A4 expression and activation via PXR dependent pathways, and might contribute to drug-drug interactions”. As taken from Zhang YW et al. 2014. *Pharmazie* 69(7), 532-6. PubMed, 2015

Type of Test	Route of Exposure or Administration	Species/ Test System	Dose Data	Toxic Effects	Reference
ICLo - Inhibitor Concentration Low	In vitro	Rabbit - myocard	10 umol/L	In Vitro Toxicity Studies - membrane currents Nutritional and Gross Metabolic - changes in calcium	JOETD7 Journal of Ethnopharmacology. (Elsevier Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1-1979- Volume(issue)/page/year: 144,555,2012
IC30 - Inhibitor Concentration 30	In vitro	Rabbit - myocard	30.44 umol/L	In Vitro Toxicity Studies - membrane currents Nutritional and Gross	JOETD7 Journal of Ethnopharmacology. (Elsevier Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1-

				Metabolic - changes in calcium	1979- Volume(issue)/page/year: 144,555,2012
ICLo - Inhibitor Concentration Low	In vitro	Rabbit - myocard	40 umol/L	Cardiac - other changes In Vitro Toxicity Studies - membrane currents Nutritional and Gross Metabolic - changes in calcium	JOETD7 Journal of Ethnopharmacology. (Elsevier Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1- 1979- Volume(issue)/page/year: 144,555,2012
ICLo - Inhibitor Concentration Low	In vitro	Human - liver tumor	40 mg/L/6H	Biochemical - Metabolism (Intermediary) - lipids including transport	FCTOD7 Food and Chemical Toxicology. (Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, NY 10523) V.20- 1982- Volume(issue)/page/year: 109,930,2017
ICLo - Inhibitor Concentration Low	In vitro	Human - liver tumor	35 mg/L/12H	Biochemical - Metabolism (Intermediary) - lipids including transport	FCTOD7 Food and Chemical Toxicology. (Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, NY 10523) V.20- 1982- Volume(issue)/page/year: 109,930,2017
ICLo - Inhibitor Concentration Low	In vitro	Human - liver tumor	35 mg/L	Biochemical - Enzyme inhibition, induction, or change in blood or tissue levels - catalases Biochemical - Enzyme inhibition, induction, or change in blood or tissue levels - other oxidoreductases	FCTOD7 Food and Chemical Toxicology. (Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, NY 10523) V.20- 1982- Volume(issue)/page/year: 109,930,2017

As taken from RTECS, 2021.

Foshou San is a typical gynaecological formula with wild usage in traditional Chinese medicine. Jiawei Foshou San (JFS) is a novel ingredient prescription from Foshou San with antiendometriosis effect in unclear mechanisms. To uncover the potential application and proapoptotic mechanisms of JFS, JFS ingredient-drug target-disease networks, GO enrichment, and pathway analysis were established for potential application prediction. Molecular docking and validation in vivo were investigated by the proapoptotic mechanisms of JFS. In this study, 99 common targets were related to 108 diseases. 484 biological processes, 44 cell components, 59 molecular functions, and 37 pathways were significantly identified in GO enrichment and pathway analysis. In molecular docking, ligustrazine showed binding activity with Bcl-2, Bax, caspase-9, caspase-3, and PARP. In vivo, JFS elevated the shrink rate of ectopic endometrium, by suppressing E2 and PROG. An in-

depth study showed that apoptosis was activated through diminishing Bcl-2, rising Bax and Bad, and expressing more caspase-3 and caspase-9 using JFS. JFS promoted the protein level of cleaved-PARP. In brief, JFS might be applied for various diseases through multiple targets and pathways, especially endometriosis by Bcl-2 pathway. These findings reveal the potential application for further evaluation and uncover the proapoptotic mechanism of JFS.” As taken from Wei J et al. 2019. Evid. Based Complement. Alternat. Med. 2019, 2360367. PubMed, 2020

“Tetramethylpyrazine (TMPZ), a Chinese herb effectively used for cardiovascular disorders. The present study reports in vitro interaction studies of TMPZ with human serum albumin (HSA) using various spectroscopic, isothermal titration calorimetric (ITC), molecular modelling studies. The association constant (K_a), standard enthalpy change (ΔH°), standard entropy change (ΔS°) and standard Gibbs free energy change (ΔG°) were obtained from ITC. The thermodynamic investigations suggest that the interaction of HSA and TMPZ is an exothermic process and enthalpy driven. Far-UV CD spectroscopy has been applied to check the stability of HSA in presence of TMPZ under thermal and chemical denaturant condition. Molecular docking was performed to confirm the binding site of TMPZ on HSA and amino acids involved in the binding process and it is found that TMPZ binds at Sudlow site I of HSA. Comparative MD simulation study of HSA and HSA-TMPZ complex shows that the structure of HSA in both systems is stable and has almost similar properties. The binding free energies calculated from the molecular mechanics/Poisson-Boltzmann surface area (MM-PBSA) method showed that van der Waals forces are the predominant intermolecular forces. Esterase-like activity of HSA decreases in the presence of TMPZ. Thus, this study will provide useful information regarding the interaction of TMPZ with HSA, helping to understand the activity and mechanism of drug binding and highlights its importance in the clinical medicine.” As taken from Khatun S et al. 2020. The Journal of Chemical Thermodynamics 140, 105915.

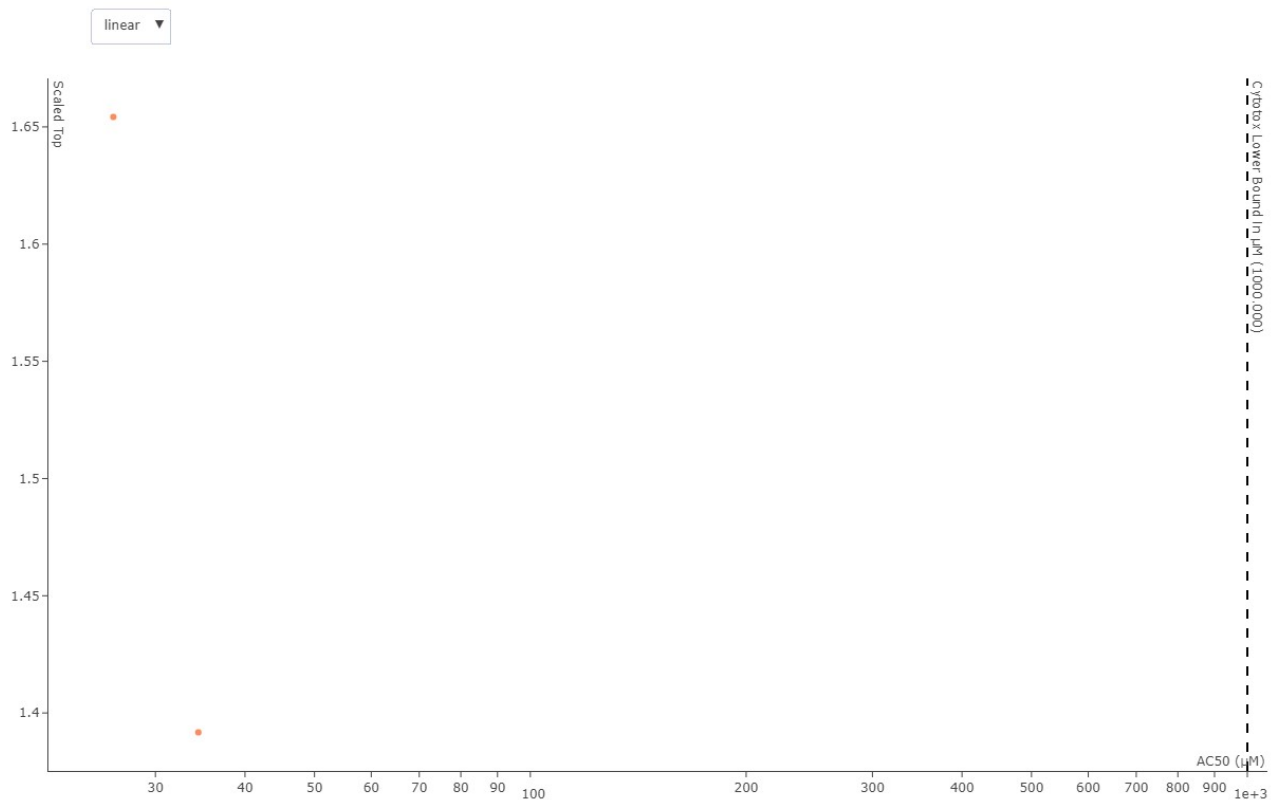
High-throughput Assay Data

The US Environmental Protection Agency (EPA) evaluated tetramethylpyrazine in a series of high-throughput assays, which are publicly available on the US EPA's CompTox Dashboard (section BIOACTIVITY / sub-section TOXCAST:SUMMARY), available at the following URL: <https://comptox.epa.gov/dashboard>

US EPA provides the following data use considerations for ToxCast data: “The activity of a chemical in a specific assay does not necessarily mean that it will cause toxicity or an adverse health outcome. There are many factors that determine whether a chemical will cause a specific adverse health outcome. Careful review is required to determine the use of the data in a particular decision contexts. Interpretation of ToxCast data is expected to change over time as both the science and analytical methods improve.”

A summary of the ToxCast assay data on tetramethylpyrazine is provided below in Figure 1. Figure 1 proves an overview of the types of assays where activity was noted with this substance. The complete study details are available on US EPA's CompTox Dashboard.

Figure 1



“Objective: To systematically review the efficacy and safety of Ligustrazine in the treatment of idiopathic pulmonary fibrosis (IPF). Methods: The electronic literature databases (PubMed, EMBase, CNKI, WanFang database, and VIP) were retrieved through a computer to find out the randomized controlled trials (RCT) of Ligustrazine in the treatment of IPF according to the inclusion/exclusion criteria screening test. Cochrane's bias risk table was also used to evaluate the quality of the study and to extract effective data. RevMan 5.3 was used for statistical analysis. Results: A total of 7 RCTs (a total of 366 patients, including 196 in experimental and 170 in control group). Compared with the control group, Ligustrazine could improve the clinical symptoms ([OR] = 2.20, 95% CI [1.40, 3.46], P=0.0006), lung function (VC % [MD] = 3.92, 95% CI [0.68, 7.17], P=0.02), (TLC% [MD] = 4.94, 95% CI [0.37, 9.52], P=0.03), the pulmonary diffusion function (DLCO % [MD] = 9.12, 95% CI [5.70, 12.55], P < 0.00001), and arterial blood gas analysis (PaO₂ [MD] = 7.11, 95% CI [1.96, 12.25], P=0.007) (PaCO₂ [MD] = -2.42, 95% CI [-4.36, -0.49], P=0.01) of IPF patients, respectively. However, FEV₁/FVC % ([MD] = 9.37, 95% CI [-1.23, 19.97], P=0.08) and adverse reactions ([MD] = 0.35, 95% CI [0.02, 5.36], P=0.45) were not significantly improved. Conclusion: Ligustrazine has certain clinical efficacy in the treatment of IPF, but the safety of applying it and the adverse reactions need to be further analyzed and determined. It can be considered as a new alternative and complementary medicine to be promoted and recommended for use in medical units in various countries in the world and it solved the difficult problem of conventional drug treatment of IPF; therefore, more research strength can be put in the treatment of the pathological mechanism of IPF for further exploration.” As taken from Wu X et al. 2020. Evid. Based Complement. Alternat. Med. 2020, 2416132. PubMed, 2021

6. Functional effects on

6.1. Broncho/pulmonary system

“To study the protective effect of tetramethylpyrazine (TMP) on the respiratory inhibition induced by hypoxia. METHODS: Forty-four SD rats were used. The hypoxic rat model was established. The

effects of TMP on the responses of respiratory activity and life time to hypoxia were observed. The Nissl's staining and immunohistochemical technique were used to investigate the effect of hypoxia on the Nissl's bodies and FOS expression in the brainstem and the antagonistic action of TMP. In contrast to the group of hypoxia, the occurrence of respiratory inhibition was delayed and the life time was prolonged obviously in the rats of hypoxia plus TMP ($P<0.01$). Compared with the air control group, the intensity of the Nissl's staining of the brainstem neurons became weaker in the group of hypoxia, but in the group of hypoxia plus TMP, the Nissl's staining was stronger and the gray degree decreased obviously, compared with the group of hypoxia ($P<0.05$). FOS positive neurons were induced by hypoxia and mainly existed in the nucleus of solitary tract, area postrema, hypoglossal nucleus, lateral reticular nucleus, inferior olivary nucleus, nucleus raphe pallidus, facial nucleus, trapezoid nucleus, but in the group of hypoxia plus TMP, the level of FOS immunoreactivity decreased remarkably, compared with the group of hypoxia ($P<0.05$). TMP has protective effect on the respiratory inhibition induced by hypoxia and FOS may be involved in the protection." As taken from Li L et al. Sichuan Da Xue Xue Bao Yi Xue Ban. 2005. 36(4), 533-6

"Acute lung injury is a common complication in patients with extensive burns in which the burned area exceeds 30% of the total body surface area (TBSA). This study was undertaken to evaluate the effect of Ligustrazine on burn-induced lung injury as well as the release of interleukin-8 (IL-8) in rats to characterize the role of Ligustrazine and IL-8 in lung injury after burn trauma. Sprague-dawley rats were divided into three groups: (1) sham group, rats who underwent sham burn; (2) control group, rats given third-degree burns over 30% TBSA and lactated Ringer solution for resuscitation; and (3) Ligustrazine group, rats given burn injury and lactated Ringer's solution with Ligustrazine inside for resuscitation. Pulmonary injury was assessed at 24 h by pulmonary capillary permeability determined with fluorescein isothiocyanate-labeled albumin and lung histologic analysis, and lung myeloperoxidase (MPO) activity as well as lung wet/dry weight ratio. The IL-8 levels were measured in serum by enzyme-linked immunosorbent assay. These studies showed that burn trauma results in increased pulmonary leakage permeability and lung wet/dry ratio, elevated serum IL-8 levels and MPO activity, and worsened histologic condition. Ligustrazine inhibited these changes, prevented burn-mediated lung injury, and the production of IL-8. This will likely provide further evidence for ligustrazine as a therapeutic strategy in burn-induced lung injury." As taken from Zheng H et al. Burns. 2005. 31(4):453-8. PubMed, 2010

"Organ protection is a routine therapy in severe burn/scald injuries, and damage mechanisms following early scald injury was not been fully elucidated. Our aim was to verify the beneficial effects of Ligustrazine on pulmonary damage associated with scald injury. Lewis rats were subjected to 30% total body surface area (TBSA) scald injury, and were randomly divided into a burn control (S group) and an Ligustrazine-treated group (L group). Lung malondialdehyde (MDA) and superoxide dismutase (SOD) levels were determined and the lungs were examined histologically with immunohistochemistry (IHC) as well for the MHC class I chain-related antigen A (MICA) and Bcl-2 at 24, 48 and 72h after the injury. The expression of spleen HLA-DR was detected by immunohistochemistry analysis. Selectins and adhesion molecules in lungs and serum as well as pulmonary interleukins were also detected. The lung injury degree was represented as wet/dry (W/D) values and alveolar thickness. Ligustrazine decreased MDA levels and ameliorated the down-regulation of SOD activity. MICA was up-regulated after the scald, and this up-regulation was greatly diminished by Ligustrazine. Bcl-2 was up-regulated after the scald, especially in the L group. The spleen HLA-DR expression demonstrated the immunoregulatory effects of Ligustrazine, which effectively protected pulmonary tissues from scald-induced injury. Our results demonstrated that pulmonary damage associated with autoimmunity and oxidant attack occurred after severe scald. Ligustrazine exhibits significant protective effects on these effects." As taken from Gao C et al. Burns. 2012. 38(5):743-50. PubMed, 2013

"We focused on the production and evaluation of a modified "double-hit" induced acute lung injury (ALI) model, which closely mimics the clinical situation. Further, tetramethylpyrazine (TMP), an alkaloid contained in ligustrazine was evaluated for its potent anti-inflammatory effects in this model. Rats were randomized into 4 groups: G1 (NS control group), G2 ("double-hit" group), G3

(low dosage TMP group) and G4 (high dosage TMP group). The rats in G2, G3 and G4 were intraperitoneally injected with a low dose of LPS followed by intratracheal injection with median dose of LPS to establish the "double-hit" model. The rats in G3, G4 were intraperitoneally injected with low (G3), high (G4) dosage TMP for the protection against ALI. Upon termination of the experiment, TMP attenuated the harmful changes in animal model reaction, breathing frequency, histological examination, lung W/D-weight ratio, BAL fluid PMNs percentage, MPO activity and ROCK2 mRNA expression. We found inhibiting RhoA/ROCK pathway might attribute to TMP-induced protection against ALI." As taken from Zhao S et al. 2015. *Int. J. Clin. Exp. Pathol.* 8(5), 4581-7. PubMed, 2016

"We investigated the effectiveness of ligustrazine (tetramethylpyrazine, TMP) in alleviating pulmonary damage induced by lipopolysaccharide (LPS). Twenty-four healthy male Sprague-Dawley rats were randomly divided into three groups: the blank group, LPS group, and TMP treatment group (TMP group). The LPS group was intraperitoneally injected with LPS (20mg/kg), and the TMP group was intraperitoneally injected with LPS (20mg/kg) and ligustrazine (80mg/kg). Blood gas analysis, hematoxylin-eosin staining dye extravasation and diffuse alveolar damage (DAD) score, the wet/dry lung tissue (W/D) ratios, the expression of CD31+ vascular endothelial microparticles (EMPs), tumor necrosis factor alpha (TNF- α) levels, and the protein expression of Rho-associated coiled-coil-forming protein kinase (ROCK) II and Toll-like receptor 4 (TLR4) were analyzed. Compared with the blank group, the arterial partial pressure of oxygen (PaO₂) declined in both 1 and 4h ($P<0.01$), the W/D ratio and DAD score increased ($P<0.01$), and the TNF- α levels in serum, CD31+ EMPs, and protein expression of ROCK II and TLR4 were significantly increased ($P<0.01$) in the LPS group. Compared with the LPS group, PaO₂ significantly increased in the TMP group at 4h ($P<0.05$), while the W/D ratio and DAD score were significantly decreased in the TMP group ($P<0.01$). TNF- α levels, CD31+ EMPs, and protein expression of ROCK II and TLR4 were significantly decreased in the TMP group compared with the LPS group ($P<0.01$). This study demonstrated that TMP can alleviate LPS-induced pulmonary damage by attenuating pulmonary vascular permeability and CD31+ EMP levels in the plasma, reducing the release of the inflammatory mediator TNF- α and inhibiting the protein expression of ROCK II and TLR4." As taken from Wang H et al. 2015. *Burns* 41(6). 1235-41. PubMed, 2016

"OBJECTIVE: To investigate the effects of ligustrazine (LTZ) on airway inflammation in a mouse model of neutrophilic asthma (NA). METHODS: Forty healthy C57BL/6 female mice were randomly divided into 4 groups using a random number table, including the normal control, NA, LTZ and dexamethasone (DXM) groups, with 10 rats in each group. The NA mice model was established by the method of ovalbumin combined with lipopolysaccharide sensitization. At 0.5 h before each challenge, LTZ and DXM groups were intraperitoneally injected with LTZ (80 mg/kg) or DXM (0.5 mg/kg) for 14 d, respectively, while the other two groups were given the equal volume of normal saline. After last challenge for 24 h, the aerosol inhalation of methacholine was performed and the airway reactivity was measured. The bronchoalveolar lavage fluid (BALF) was collected. The Wright-Giemsa staining was used for total white blood cells and differential counts. The levels of cytokines interleukin (IL)-17 and IL-10 were detected by enzyme-linked immunosorbent assay. The pathological change of lung tissue was observed by hematoxylin eosin staining. RESULTS: The airway responsiveness of the NA group was significantly higher than the normal control group ($P<0.05$), while those in the LTZ and DXM groups were significantly lower than the NA group ($P<0.05$). The neutrophil and eosinophil counts in the LTZ and DXM groups were significantly lower than the NA group ($P<0.05$), and those in the LTZ group were significantly lower than the DXM group ($P<0.05$). There were a large number of peribronchiolar and perivascular inflammatory cells in infiltration in the NA group. The airway inflammation in the LTZ and DXM groups were significantly alleviated than the NA group. The infiltration in the LTZ group was significantly reduced than the DXM group. Compared with the normal control group, the IL-17 level in BALF was significantly increased and the IL-10 level in BALF was significantly decreased in the NA group ($P<0.05$). LTZ and DXM treatment significantly decreased IL-17 levels and increased IL-10 levels compared with the NA group ($P<0.05$), and the changes in the above indices were more

significant in the LTZ group ($P < 0.05$). CONCLUSION: LTZ could alleviate the airway inflammation in the NA mice model through increasing the IL-10 level and decreasing the IL-17 level." As taken from Liu XM et al. 2018. Chin. J. Integr. Med. 24(5), 353-358. PubMed, 2019

"Asthma is an inflammatory disease which affects millions of people worldwide. Therefore, it is necessary to search for new sources of therapies for the treatment of these patients in order to improve their quality of life. From content analysis of literature of new therapeutic targets, there are various targets and drugs reported to be promising for the treatment of asthma. Interleukins involved in inflammatory processes are often presented as candidate targets for new drugs. The action of such therapeutics would not only affect interleukins, but also their receptors. Small molecules (e.g. ligustrazine and SP600125) and large molecule antibodies (e.g. lebrikizumab, benralizumab, dupilumab) are being considered as novel agents for the pharmacotherapy of asthma. Therefore, through this research, we can see advances in the search for new targets and promising drugs to treat asthma. It is expected that these new drug candidates will eventually be approved and marketed so that asthma patients can use them to enhance their quality of life." As taken from Alves MF et al. 2018. Mini Rev. Med. Chem. 18(8), 684-696. PubMed, 2019

Background: Tetramethylpyrazine (TMP), a potent anti-free radical and anti-inflammations substance, has been demonstrated to possess a direct vessel relaxation property. This study aimed to evaluate the effect of TMP treatment in pulmonary hypertension (PH) and test the hypothesis that TMP prevents or reverses the process of PH.

Methods: Rats ($n = 36$) injected with 50 mg/kg of monocrotaline (MCT) subcutaneously 4 weeks to develop PH were then randomized to TMP (5 mg/kg per day) for another 4 weeks. Hemodynamics was evaluated via the right ventricle. Pulmonary vessels structural remodeling and inflammation were examined by histologic and transmission electron microscopy observation. The expression of inducible nitric oxide synthase (iNOS) and cGMP-dependent protein kinases 1 (PKG-1) was detected by immunohistochemical staining and Western blot. Generation of reactive oxygen species (ROS) and antioxidation species was measured by biochemical analyses.

Results: MCT increased PH and right ventricle hypertrophy. TMP alleviated pulmonary arterial pressure elevation, leukocyte infiltration, and structural remodeling of pulmonary arterials induced by MCT successfully. TMP treatment significantly increased the PKG-1 expression and suppressed the iNOS expression. The activity of superoxide dismutase (SOD), glutathione peroxidase (GSH), and catalase (CAT) was significantly higher than control group, while malondialdehyde (MDA) levels were lower compared with MCT group.

Conclusion: TMP can suppress established MCT-induced PH through the ROS/iNOS/PKG axis. The underlying mechanisms may be associated with its anti-inflammatory, antioxidant, and antiproliferative properties in pulmonary arterial.

Yang, D. P., et al. (2022). Tetramethylpyrazine Improves Monocrotaline-Induced Pulmonary Hypertension through the ROS/iNOS/PKG-1 Axis.

Due to the antioxidant properties of TTMP, inhaled TTMP aerosols prevented the progression of pulmonary hypertension in a rat model. Min et al. reported that TTMP has a therapeutic role in preventing acute lung injury through protecting the pulmonary microvascular endothelial cell barrier and suppressing inflammation. As taken from Liu X et. Al. 2024. Molecules.

6.2. Cardiovascular system

"Acute p.o. administration of 99.5% ethanol (0.1 ml) to fasted mice produced heart toxicity. Pretreatment with p.o. administration of tetramethylpyrazine (TMP) could prevent such toxicity effectively and dose-dependently. The maximal antioxidative effect against 99.5% ethanol-induced heart toxicity could be observed at 1 hour after TMP administration. In order to further investigate the heart protective mechanism of TMP, both lipid peroxidation level in vivo and superoxide scavenging activity were conducted. TMP exhibited a dose-dependently anti-lipid peroxidation effect in mice heart homogenate, and results indicated that 99.5% ethanol-induced intoxicated mice

hearts have higher malonic dialdehyde (MDA) levels compared with those in TMP administrated mice hearts. These results suggest that the potentially heart protective mechanism of TMP could be contributed, at least in part, to its prominent anti-lipid peroxidation and anti-free radical formation effects, hence it could protect the heart from lipid peroxidation-induced heart toxicity.” As taken from Liu CF et al. *Am. J. Chin. Med.* 2005. 33(6):981-8. PubMed, 2010

“2,3,5,6-Tetramethylpyrazine (No. 780) inhibited platelet aggregation in platelet-rich plasma from blood obtained from healthy volunteers. An increased number of alkyl groups on the pyrazine ring or increased length of the unbranched alkyl side-chains enhanced the antiplatelet activity of the tetramethylpyrazine analogues tested. The alkoxyl derivatives tested were less potent inhibitors than alkyl derivatives with the same number of carbons on pyrazine. The inhibition of platelet aggregation correlated with the lipophilicity of the test substance (Liu & Sylvester, 1994).”

As taken from JECFA, 2002.

“Tetramethylpyrazine (TMP) is the biologically active ingredient isolated from a popular Chinese medicinal plant, *Ligusticum wallichii* franchet, which has been used effectively since the 1970s to treat ischemic heart disease, cerebrovascular and thrombotic vascular diseases. The direct action of TMP on cardiac contractile function, however, is largely unclear. This study was designed to examine the effect of TMP on ventricular contractile function at the single cardiac myocyte level. Adult rat ventricular myocytes were isolated and stimulated to contract at 0.5 Hz, and mechanical and intracellular Ca^{2+} properties were evaluated using an IonOptix Myocam system. Contractile properties analyzed included peak shortening (PS), time-to-peak shortening (TPS), time-to-90% relengthening (TR(90)), maximal velocity of shortening/relengthening ($\pm$ -dl/dt), resting intracellular Ca^{2+} level, Ca^{2+} -induced Ca^{2+} release (CICR) and decay. TMP (10(-10)-10(-5) M) exhibited an increase in PS with a maximal increase of 30.9%. TMP had no effect on $\pm$ -dl/dt, TPS/TR(90) or CICR but lowered resting intracellular Ca^{2+} level and slowed intracellular Ca^{2+} decay. Pretreatment with either the nonspecific nitric oxide synthase (NOS) inhibitor Nomega-nitro-L-arginine methyl ester (L-NAME, 100 microM) or inducible NOS inhibitor W1400 effectively abolished the positive effect of TMP on myocyte shortening. Our data demonstrate a direct positive inotropic effect of TMP in cardiac myocytes, which may be related, at least in part, to NO production.” As taken from Hintz KK, Ren J. *Vascul. Pharmacol.* 2003. 40(4), 213-7. PubMed, 2010

“In this study, tetramethylpyrazine (TMPZ) was effective in reducing the mortality of ADP-induced acute pulmonary thromboembolism in mice when administered intravenously at doses of 40 and 80 microg/g. In addition, intravenous injection of TMPZ (10 microg/g) significantly prolonged the bleeding time by approximately 1.5-fold compared with normal saline in severed mesenteric arteries of rats. Continuous infusion of TMPZ (1 microg/g per min) for 10 minutes also significantly increased the bleeding time approximately 1.6-fold, and the bleeding time returned to baseline within 60 minutes after cessation of TMPZ infusion. On the other hand, platelet thrombi formation was induced by irradiation of mesenteric venules with filtered light in mice pre-treated intravenously with fluorescein sodium (10 microg/kg). When it was intravenously injected, TMPZ (250 microg/g) significantly prolonged the latent period of the induction of platelet plug formation in mesenteric venules. TMPZ (250 microg/g) prolonged occlusion time approximately 1.4-fold (183 $\pm$ 18 seconds) compared with that of normal saline (132 $\pm$ 14 seconds). Furthermore, aspirin (300 microg/g) showed similar activity in the prolongation of occlusion time in this experiment. In conclusion, these results suggest that TMPZ has effective antithrombotic activity in vivo and may be a potential therapeutic agent for arterial thrombosis but must be assessed further for toxicity.” As taken from Sheu JR et al. *Int. J. Hematol.* 2001. 73(3), 393-8. PubMed, 2010

“The vasodilating effects of tetramethylpyrazine, the alkaloid of *Ligusticum wallichii*, in the treatment of 487 patients with cardiovascular or cerebrovascular diseases are described. The drug passed through the blood-brain barrier readily, and its concentration in the brain stem was 4 times higher than in the cerebral cortex.” As taken from Guo SK et al. *Planta Med.* 1983. 47(2), 89. PubMed, 2010

"Tetramethylpyrazine (TMP) is one of the active ingredients of the Chinese herb *Ligusticum wallichii* Franchat. It is well documented that TMP exerts a cardiovascular protective effect. The aims of the present study were to examine whether TMP alters angiotensin (Ang) II-induced endothelin (ET)-1 gene expression and to identify the putative underlying signalling pathways in vascular endothelial cells. 2. Cultured vascular endothelial cells were pre-incubated with TMP, stimulated with AngII and ET-1 gene expression was then examined. The effects of TMP pretreatment on AngII-induced extracellular signal-regulated kinase (ERK) phosphorylation were investigated to elucidate the intracellular mechanism responsible for the effects of TMP on ET-1 gene expression. 3. Tetramethylpyrazine inhibited AngII-induced ET-1 gene expression, as revealed by northern blotting and a promoter activity assay. Tetramethylpyrazine also inhibited the AngII-induced increase in intracellular reactive oxygen species (ROS), as measured by the redox sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate and ERK phosphorylation. 4. In summary, we have demonstrated, for the first time, that TMP inhibits AngII-induced ROS generation, ERK phosphorylation and ET-1 gene expression in vascular endothelial cells. Thus, the present study delivers important new insights into the molecular pathways that may contribute to the proposed beneficial effects of TMP in the cardiovascular system." As taken from Lee WS et al. Clin. Exp. Pharmacol. Physiol. 2005. 32(10), 845-50. PubMed, 2010

"To study the effects of tetramethylpyrazine (TMP) on cardiac function and mortality rate in septic rats, fifty male Sprague-Dawley rats were randomized into a sham-operation group (sham group, n=10), normal saline group (NS group, n=20), and TMP group (n=20). The rats in the NS and TMP groups underwent cecal ligation and puncture (CLP) to induce sepsis. Rats in the NS group were injected with NS (10 mL/kg) immediately after CLP and 6 h after CLP. Rats in the TMP group were injected with TMP (10 mg/kg) at the same time points. Twenty-four hours after modeling, the mortality rates were observed in each group. Cardiac function and serum concentration of tumor necrosis factor α (TNF- α) were also tested. The correlation between TNF- α and the ejection fraction (EF) was observed. Left ventricle specimens were reserved for histomorphologic study. Compared with the sham group, the NS and TMP groups had decreased EF values and increased mortality rates and serum TNF- α levels ($P < 0.05$). The TMP group had a comparatively lower mortality rate and TNF- α level and a higher EF value compared with the NS group ($P < 0.05$). Histomorphology indicated that myocardial inflammation in the TMP group was mild compared with that in the NS group. There was a negative correlation between TNF- α level and EF value ($r = -0.583, P = 0.000$). TMP could reduce the mortality rate of septic rats and had certain protective effects on cardiac function." As taken from Guo LH et al. Chin. J. Integr. Med. 2012. 18(8), 610-5. PubMed, 2013

"A series of novel 2-acyloxymethyl-3,5,6-trimethylpyrazine derivatives was designed and synthesized. Most compounds were found to be 1.5-4.5-fold higher potency than tetramethylpyrazine (TMP) in stimulating the proliferation of normal vascular endothelial cells and in protecting against hyperoxic acute injury. The most active one is the 2-nicotinoyl ester 5a exhibiting the maximum proliferation rate ($P(\max)$) of 88.57% at the concentration of 0.1 mmol L⁻¹. Structure-activity relationships of these compounds were discussed." As taken from Liu X et al. Bioorg. Med. Chem. Lett. 2003. 13(13), 2123-6. PubMed, 2013

"We assessed whether tetramethylpyrazine (TMP), an active ingredient of *Ligusticum wallichii* Franchat, attenuates atherosclerosis (AS) development in rabbits and protects endothelial cells injured by ox-LDL. METHODS: In vivo, rabbits subjected to atherosclerosis were treated with TMP (75 and 150 mg/kg) by oral gavage for 12 weeks. In vitro, rat aortic endothelial cells (RAECs) were stimulated by ox-LDL. RESULTS: TMP treatment with 75 and 150 mg/kg significantly reduced the relative atherosclerosis area ratio in the aorta ($0.41 \pm 0.042, 0.27 \pm 0.047$ vs. 0.66 ± 0.058 in AS), the ratio of intimal/medial thickness ($0.54 \pm 0.09, 0.39 \pm 0.07$ vs. 1.1 ± 0.3 in AS) and the number of monocytes in intimal ($10.1 \pm 2.8, 8.2 \pm 2.0$ vs. 14.1 ± 4.9 counts/mm² in AS). TMP also decreased levels of TC (15 ± 4.2 to 6.1 ± 1.2 mmol/L), TG (1.8 ± 0.3 to 1.08 ± 0.24 mmol/L), LDL-C (20.1 ± 4.3 to 10.2 ± 1.6 mmol/L) and increased HDL-C levels (0.40 ± 0.08 to 0.85 ± 0.17 mmol/L) in

atherosclerosis rabbit plasma. TMP decreased the MCP-1 (187.3 ± 38.4 to 86.1 ± 17.2 pg/ml) and ICAM-1 (350.6 ± 43.7 to 260.6 ± 46.1 pg/ml) levels in plasma and inhibited LOX-1 expression in the rabbit aortas. Moreover, our in vitro study revealed that TMP suppressed monocyte adhesion to RAECs, inhibited RAEC migration, and down-regulated MCP-1 and ICAM-1 expression in ox-LDL-injured RAECs. Likewise, TMP inhibited LOX-1 and 5-LOX expression, and prevented nuclear accumulation of RelA/p65 and I κ B degradation in ox-LDL-injured RAECs. Furthermore, TMP suppressed ox-LDL-induced activations of p-ERK, p-p38, and p-JNK MAPK. CONCLUSION: TMP produces a tangible protection in atherosclerosis and endothelial cells. TMP might be a potential protective agent for atherosclerosis." As taken from Wang GF et al. 2013. *Cardiovasc. Drugs Ther.* 27(3), 199–210. PubMed, 2013

"Tetramethylpyrazine (TMP), a bioactive compound isolated from the Chinese herb, *Ligusticum wallichii* Franchet, has been reported to play a protective role in cardiac diseases. However, the cellular and molecular mechanisms behind the protective effects of TMP on the heart remain to be elucidated. In this study, we aimed to determine the effects of TMP on angiotensin II (Ang II)-induced hypertrophy in neonatal rat cardiomyocytes and its possible mechanisms of action. In addition, we investigated whether TMP regulates tumor necrosis factor- α (TNF- α) secretion and expression. We found that TMP significantly inhibited the Ang II-induced hypertrophic growth of neonatal cardiomyocytes, as evidenced by the decrease in [3H]leucine incorporation and β -myosin heavy chain (β -MHC) mRNA expression. TMP inhibited Ang II-stimulated TNF- α protein secretion and mRNA expression in the cardiomyocytes. Further experiments revealed that Ang II increased the level of the phosphorylated form of the transcription factor, nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B), as well as NF- κ B-DNA binding activity in the cardiomyocytes; treatment with TMP significantly inhibited the Ang II-induced activation of NF- κ B. Furthermore, the inhibition of NF- κ B by the specific inhibitor, pyrrolidine dithiocarbamate (PDTC), markedly attenuated the Ang II-induced increase in [3H]leucine incorporation, β -MHC mRNA expression and TNF- α protein secretion. Our findings suggest that TMP inhibits Ang II-induced cardiomyocyte hypertrophy and TNF- α production through the suppression of the NF- κ B pathway, which may provide new insight into the mechanisms underlying the protective effects of TMP in heart diseases." As taken from Yu L et al. 2013. *Int. J. Mol. Med.* 32(3), 717–22. PubMed, 2013

"OBJECTIVE: To study the action mechanism of tetramethylpyrazine (TMP) on the proliferation of vascular smooth muscle cells (VSMCs), thus providing experimental evidence for Chinese medicing to effectively prevent restenosis. METHODS: Rats' thoracic aorta VSMCs in vitro cultured (cell line A7r5) were divided into five groups, i.e., the negative control group, the angiotensin II (Ang II, $10(-6)$ mol/L) group, the low dose TMP (20 micromol/L) plus Ang II group, the middle dose TMP (40 micromol/L) plus Ang II group, the high dose TMP (80 micromol/L) plus Ang II group. The proliferation ratio was detected by MTT. Gene and protein expressions of Wnt4, Dvl-1, beta-catenin, CyclinD1, and collagen I and III were detected with real-time fluorescent quantitative PCR and Western blot respectively. RESULTS: Compared with the negative control group, the proliferation ratio of VSMCs obviously increased in the Ang II group ($P < 0.05$). Compared with the Ang II group, the proliferation ratio of VSMCs obviously decreased in the middle dose TMP plus Ang II group and the high dose TMP plus Ang II group ($P < 0.05$). Compared with the negative control group, gene and protein expressions of Wnt4, Dvl-1, beta-catenin, CyclinD1, Col I, and Col III were obviously up-regulated in the Ang II group ($P < 0.05$). Compared with the Ang II group, mRNA and protein expressions of Wnt4, Dvl-1, beta-catenin, CyclinD1, Col I, and Col III were obviously down-regulated in the middle dose TMP plus Ang II group and the high dose TMP plus Ang II group ($P < 0.05$). The aforesaid indices were dose-dependent in the low, middle, and high

dose TMP plus Ang II groups. CONCLUSION: TMP inhibited Ang II induced proliferation and collagen secretion of VSMCs through down-regulating Wnt signal pathway.” As taken from Hua JY et al. 2013. *Zhongguo Zhong Xi Yi Jie He Za Zhi*. 33(9), 1226-31. PubMed, 2013

“Atherosclerosis (AS) is the leading cause of death in modern societies. Active substance from Traditional Chinese Medicine has been used for the treatment of AS, such as ligustrazine and puerarin. However, the pathogenesis of AS and the curative mechanisms of ligustrazine and puerarin stay unclear. In this work, we attempted to figure out these questions using a rat AS model and digital gene expression (DGE) system. Our results showed that DGE sequencing outcomes were high quality and reproductively. Differentially expressed genes were obtained from different comparisons. The Gene Ontology (GO) analysis revealed that mainly enriched GO terms due to the drug treatment were the same as those obtained from the control group vs. the AS model group. Pathway analysis indicated that metabolic pathways, oxidative phosphorylation, and PPAR single pathways were enriched in all comparisons. Our work provided a comprehensive basis for a better understanding of the pathogenesis of AS and the curative mechanisms of ligustrazine and puerarin”. As taken from Fu R et al. 2014. *Gene* 552(1), 75-80. PubMed, 2015

“Tetramethylpyrazine (TMP) is an active compound isolated from a Chinese herbal prescription that is widely used in traditional Chinese medicine for the treatment of inflammatory and cardiovascular diseases. We have previously reported that TMP acts as a potent antioxidant protecting endothelial cells against high glucose-induced damages. However, the molecular mechanism responsible for the antioxidant effect of TMP remains to be elucidated. In this study, we show that TMP increases nitric oxide production in endothelial cells and promotes endothelium-dependent relaxation in rat aortic rings. The antioxidant effect of TMP appears attributable to its ability to activate the mitochondrial biogenesis, as reflected in an up-regulation of complex III and amelioration of mitochondrial membrane potential. Furthermore, TMP is able to reverse high glucose-induced suppression of SIRT1 and the biogenesis-related factors, including PGC-1 α , NRF1 and TFAM, suggesting a new molecular mechanism underlying the protective effect of TMP on the endothelium”. As taken from Xu Q. 2014. *PLoS One* 9(2), e88243. PubMed, 2015

“Tetramethylpyrazine (TMP) is one of the active ingredients extracted from the Chinese herb Chuanxiong, which has been used to treat cerebrovascular and cardiovascular diseases, pulmonary diseases and cancer. However, the molecular mechanisms underlying the actions of TMP have not been fully elucidated. In a previous study we showed that TMP-mediated glioma suppression and neural protection involves the inhibition of CXCR4 expression. The SDF-1/CXCR4 axis plays a fundamental role in many physiological and pathological processes. In this study, we further investigated whether the regulation of the SDF-1/CXCR4 pathway is also involved in the TMP-mediated inhibition of neovascularization or fibrosis and improvement of microcirculation. METHODOLOGY/PRINCIPAL FINDINGS: Using a scratch-wound assay, we demonstrated that TMP significantly suppressed the migration and tubule formation of the human umbilical vein endothelial cell line ECV304 in vitro. The expression of CXCR4 in ECV304 cells is notably down-regulated after TMP treatment. In addition, TMP significantly suppresses corneal neovascularization in a rat model of corneal alkali burn injury. The expression of CXCR4 on days 1, 3 and 7 post-injury was determined through RT-PCR analysis. Consistent with our hypotheses, the expression of CXCR4 in the rat cornea is significantly increased with alkali burn and dramatically down-regulated with TMP treatment. Moreover, TMP treatment significantly attenuates bleomycin-induced rat pulmonary fibrosis, while immunofluorescence shows a notably decreased amount of CXCR4-positive cells in the TMP-treated group. Furthermore, TMP significantly down-regulates the expression of CXCR4 in platelets, lymphocytes and red blood cells. Whole-blood viscosity and platelet aggregation in rats are significantly decreased by TMP treatment. CONCLUSIONS: These results show that TMP exerts potent effects in inhibiting neovascularization, fibrosis and thrombosis under pathological conditions; thus, the underlying mechanism of TMP might partially contribute to the down-regulation of CXCR4”. As taken from Cai X et al. 2014. *PLoS One* 9(2), e88176. PubMed, 2015

“BACKGROUND: Hemorrhagic shock (HS) is the major cause of death from trauma. Hemorrhagic shock may lead to cellular hypoxia and organ damage. Our previous findings showed that HS induced a cardiac apoptosis pathway and synergistically caused myocardial cell damage in diabetic rats under trauma-induced HS. Tetramethylpyrazine (TMP) is a major biologically active ingredient purified from the rhizome of *Ligusticum wallichii* (called Chuang Xiong in Chinese). Chuang Xiong rescued cells from synergistic cardiomyoblast cell injury under high-glucose (HG) conditions plus hypoxia. TMP is one of the most important active ingredients that elevated the survival rate in ischemic brain injury and prevented inducible NO synthase expression to have anti-inflammatory effects against cell damage in different cell types. METHOD: Here, we further investigate whether TMP can protect against hypoxic (<1% oxygen) conditions in H9c2 cardiomyoblast cells for 24 hrs. RESULTS: Our results showed that hypoxia mediated through HIF-1 α /JNK/p38 activation significantly elevated the levels of the hypoxia-related proteins HIF-1 α , BNIP3 and IGFBP3, further enhanced the pro-apoptotic protein Bak and upregulated downstream Caspase 9 and 3, resulting in cell death. All of these phenomena were fully recovered under TMP treatment. We observed that TMP exerted this effect by activating the IGF1 receptor survival pathway, dependent primarily on PI3K/Akt. When PI3K (class I) was blocked by specific siRNA, the hypoxia-induced activated caspase 3 and cell apoptosis could not be reversed by TMP treatment. CONCLUSION: Our results strongly suggest that TMP could be used to restore hypoxia-induced myocardial cell apoptosis and cardiac hypoxic damage.” As taken from Lin KH et al. 2015. *Cell. Physiol. Biochem.* 36(1), 334-44. PubMed, 2016

“OBJECTIVE: To investigate the inhibitory effect of tetramethylpyrazine (Tet) preconditioning on overload training-induced myocardial apoptosis in rats, and to explore cardioprotective mechanisms of Tet preconditioning. METHODS: A total of 25 male Sprague-Dawley rats were randomly divided into three groups, including the control group (n=5), the overload training group (overload training for 8 weeks, n=10), and the Tet preconditioning group (Tet preconditioning for 8 weeks before overload training, n=10). After 8 weeks, cardiac structure and myocardial apoptosis were analyzed by histology, transmission electron microscopy, and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay staining. The expressions of Bcl-2, Bax, Caspase-3, and Caspase-9 in myocardium were evaluated by immunohistochemical staining. RESULTS: Overload training caused swelling, disorder, partial rupture, and necrosis of myocardial focal necrotic fibers, as well as mitochondrial vacuolization, cristae rupturing, and blurring. In contrast, Tet preconditioning attenuated the swelling of myocardial fibers, decreased the amount of ruptured fibers, and inhibited mitochondrial vacuolization, resulting in clear cristae. Overload training significantly increased Bax expression and decreased Bcl-2/Bax ratio when compared with the control group ($P<0.01$). Conversely, Tet preconditioning significantly increased Bcl-2 expression and the Bcl-2/Bax ratio as compared with the overload training group ($P<0.05$). Overload training dramatically increased the expressions of Caspase-3 and Caspase-9 when compared with the control group ($P<0.05$). Following Tet preconditioning, the expression of Caspase-3 was significantly reduced compared with the overload training group ($P<0.05$), while Caspase-9 expression showed a slight decline ($P>0.05$). CONCLUSION: Tet preconditioning increased the expression of Bcl-2 and reduced the expression of Caspase-3, thereby attenuating overload training-induced myocardial apoptosis, protecting against overload training-induced myocardial injury, and reducing damage to the myocardium due to overload training.” As taken from Yang Y et al. 2015. *Chin. J. Integr. Med.* 21(6), 423-30. PubMed, 2016

“The abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) leads to intimal thickening of the aorta and is, therefore, important in the development of arteriosclerosis. As a result, the use of antiproliferative and antimigratory agents for VSMCs offers promise for the treatment of vascular disorders. Although several studies have demonstrated that ligustrazine may be used to treat heart and blood vessel diseases, the detailed mechanism underlying its actions remain to be elucidated. In the present study, the inhibitory effect of ligustrazine on platelet-derived growth factor (PDGF)-BB-stimulated VSMC proliferation and migration, and the underlying mechanisms were investigated. The findings demonstrated that ligustrazine significantly inhibited

PDGF-BB-stimulated VSMC proliferation. VSMCs dedifferentiated into a proliferative phenotype under PDGF-BB stimulation, which was effectively reversed by the administration of ligustrazine. In addition, ligustrazine also downregulated the production of nitric oxide and cyclic guanine monophosphate, induced by PDGF-BB. Additionally, ligustrazine significantly inhibited PDGF-BB-stimulated VSMC migration. Mechanistic investigation indicated that the upregulation of cell cycle-associated proteins and the activation of the extracellular signal-regulated kinase (ERK) and P38 mitogen-activated protein kinase (MAPK) signaling induced by PDGF-BB was suppressed by the administration of ligustrazine. In conclusion, the present study, demonstrated for the first time, to the best of our knowledge, that ligustrazine downregulated PDGF-BB-induced VSMC proliferation and migration partly, at least, through inhibiting the activation of the ERK and P38 MAPK signaling.” As taken from Yu L et al. 2015. Mol. Med. Rep. 12(1), 705-11. PubMed, 2016

“Ligustrazine, also known as 2,3,5,6-tetramethylpyrazine (TMP), one of the major active compounds of *Ligusticum wallichii* Franchat., has been shown to reduce neuroinflammation and protect neurons during cerebral ischemia/reperfusion injury. However, whether it reduces blood-brain barrier (BBB) permeability during ischemic stroke is unclear. The aim of the present study was to investigate the role that TMP plays in protecting the BBB integrity in ischemia/reperfusion injury and to investigate the relevant mechanisms involved. Rats received an intraperitoneal injection of 20 mg/kg TMP 15 min before the onset of ischemia, which was induced by middle cerebral artery occlusion. Infarct volume, neurological score, brain edema, BBB permeability and tight junction protein impairment were observed. The results showed that TMP reduced the neurological score and levels of brain infarction and edema. In addition, TMP significantly decreased BBB permeability and prevented the impairment of occludin and claudin-5, two tight junction protein components of the BBB, in rat brains with ischemia/reperfusion injury. In addition, the expression and activity of matrix metalloproteinases, enzymes responsible for the degradation of the extracellular matrix and tight junctions, were reduced in the rat brains by TMP treatment. These results combined suggest that TMP reduces BBB permeability as well as neuronal damage in focal cerebral ischemia/reperfusion injury in rats.” As taken from Tan F et al. 2015. Exp. Ther. Med. 9(5), 1757-1762. PubMed, 2016

Type of Test	Route of Exposure or Administration	Species/ Test System	Dose Data	Toxic Effects	Reference
ICLo - Inhibitor Concentration Low	In vitro	Rabbit - myocard	10 umol/L	In Vitro Toxicity Studies - membrane currents Nutritional and Gross Metabolic - changes in calcium	JOETD7 Journal of Ethnopharmacology. (Elsevier Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1-1979- Volume(issue)/page/year: 144,555,2012
IC30 - Inhibitor Concentration 30	In vitro	Rabbit - myocard	30.44 umol/L	In Vitro Toxicity Studies - membrane currents Nutritional and Gross Metabolic - changes in calcium	JOETD7 Journal of Ethnopharmacology. (Elsevier Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1-1979- Volume(issue)/page/year: 144,555,2012
ICLo - Inhibitor Concentration	In vitro	Rabbit -	40	Cardiac - other changes In	JOETD7 Journal of Ethnopharmacology. (Elsevier

Low		myocard	umol/L	Vitro Toxicity Studies - membrane currents Nutritional and Gross Metabolic - changes in calcium	Scientific Pub. Ireland Ltd., POB 85, Limerick, Ireland) V.1- 1979- Volume(issue)/page/year: 144,555,2012

As taken from RTECS, 2018.

"Tetramethylpyrazine (TMP), a biologically active ingredient first extracted from the Chinese medicinal plant *Ligusticum wallichii* Franchat., has athero-protective activity, yet the particular mechanisms have not been completely explored. The present study was designed to investigate the effect of TMP and its possible mechanisms in RAW264.7 macrophages and apolipoprotein E-deficient (ApoE^{-/-}) mice. TMP treatment markedly increased the cholesterol efflux and inhibited oxidized low-density lipoprotein (ox-LDL) uptake, thus, ameliorating lipid accumulation in macrophages. In addition, TMP significantly increased the protein and mRNA expression of ATP-binding cassette transporters A1 (ABCA1) and G1 (ABCG1), while suppressing the protein and mRNA expression of class A scavenger receptor (SR-A) and the cluster of differentiation 36 (CD36). Moreover, the effects of TMP on the upregulation of the expression of ABCA1 and ABCG1, the downregulation of the expression of CD36 and SR-A, the increase of cholesterol efflux and the decrease of lipid accumulation as well as the uptake of ox-LDL were mediated by the inactivation of PI3K/Akt and p38 MAPK. Furthermore, TMP upregulated the protein stability of ABCA1 without affecting ABCG1. Accordingly, TMP regulated the expression of SR-A, CD36, ABCA1 and ABCG1 in aortas of ApoE^{-/-} mice, which resembled the findings observed in macrophages. TMP was also capable of delaying the progression of atherosclerosis in ApoE^{-/-} mice. These findings revealed that TMP downregulates scavenger receptors and upregulates ATP-binding cassette transporters via PI3K/Akt and p38 MAPK signaling, thus suppressing lipid accumulation in macrophages." As taken from Duan J et al. 2017. *Oncol. Rep.* 38(4), 2267-2276. PubMed, 2018

"Lipid metabolism dysregulation plays a crucial role in the occurrence of atherosclerosis (As). SCAP/SREBP signaling is the main pathway for regulating lipid metabolism. Tetramethylpyrazine (TMP), a Traditional Chinese Medicine (TCM) for treating angina pectoris, has antiatherosclerotic effects and ameliorates blood lipids disturbance. However, its precise mechanism remains unclear. This study investigated the mechanism of TMP in ameliorating As in mice model. After six weeks of high-fat diet, 30 ApoE^{-/-} mice were randomized (n = 10) and treated with Lipitor, TMP, or distilled water for six weeks. The serum blood lipids and insulin levels were measured. The expressions of PAQR3, Insig-1, SCAP, SREBP-1c, IRS-1, PI3K, Akt, and mTORC-1 in the adipose tissues were determined. The results showed that TMP could significantly decrease blood lipids levels, insulin, and corrected plaque area of the ApoE^{-/-} mice as compared to the untreated mice ($P < 0.05$, $P < 0.01$). Moreover, TMP could significantly downregulate the expressions of SCAP, SREBP-1c, PAQR3, IRS-1, PI3K, Akt, and mTORC1 ($P < 0.01$). Thus, TMP may ameliorate lipid metabolism disorder and As by downregulating PAQR3 and inhibiting SCAP/SREBP-1c signaling pathway. In addition, PI3K/Akt/mTORC1 signaling pathway may be involved in this process." As taken from Zhang Y et al. 2017. *Evid. Based Complement. Alternat. Med.* 2017, 3121989. PubMed, 2018

Introduction: The coronary microembolization contributes to coronary microvascular dysfunction (CMD), in which miR-34a-5p may play a critical role. Ligustrazine has been reported to improve CMD. The present study was designed to discuss the role of miR-34a-5p/Sirt1 pathway in CMD and explore the underlying mechanism of ligustrazine.

Methods: Coronary microembolization (CME) was induced by left ventricle injection of sodium laurate in rats. CME formation and cardiac function were examined by HE staining and hemodynamic tests to evaluate CMD. The expressions of miR-34a-5p, Sirt1 and the downstream

proteins were detected by RT-qPCR and western blot. Dual-luciferase reporter (DLR) assay was performed to confirm the connection between miR-34a-5p and Sirt1. The blood markers of endothelial dysfunction, platelet activation and inflammation were examined with ELISA.

Results: Overt CME and cardiac dysfunction as well as up-regulated miR-34a-5p and down-regulated Sirt1 were observed in CME rats. Overexpressing miR-34a-5p aggravated while silencing miR-34a-5p inhibited CME formation. DLR assay confirmed that miR-34a-5p directly inhibited Sirt1 mRNA expression. Ligustrazine pretreatment suppressed miR-34a-5p and promoted Sirt1 expression, which alleviated endothelial dysfunction, inhibited platelet activation and inflammation, and in turn reduced CME. Overexpressing miR-34a-5p diminished the positive effects of ligustrazine; while after silencing miR-34a-5p, ligustrazine failed to further promote Sirt1 expression and inhibit CME formation.

Conclusion: MiR-34a-5p contributes to CMD by inhibiting Sirt1 expression. Ligustrazine exerts endothelial-protective, anti-platelet and anti-inflammatory effects to prevent CMD via suppressing miR-34a-5p and promoting Sirt1.

Gao, J., et al. (2022). Ligustrazine prevents coronary microcirculation dysfunction in rats via suppression of miR-34a-5p and promotion of Sirt1. Background: Atherosclerosis(AS) is widely recognized as a risk factor for incident cardiovascular and cerebrovascular diseases. Tetramethylpyrazine (TMP) is the active ingredient of *Ligusticum wallichii* that possesses a variety of biological activities against atherosclerosis.

Objective: This systematic review and meta-analysis sought to study the impact of and mechanism of tetramethylpyrazine for atherosclerosis in animal models.

Methods: A systematic search was conducted of PubMed, Embase, Cochrane Library, Web of Science database, Chinese Biomedical (CBM) database, China National Knowledge Infrastructure (CNKI), WanFang data, and Vip Journal Integration Platform, covering the period from the respective start date of each database to December 2021. We used SYRCLE's 10-item checklist and Rev-Man 5.3 software to analyze the data and the risk of bias.

Results: Twelve studies, including 258 animals, met the inclusion criteria. Compared with the control group, TMP significantly reduced aortic atherosclerotic lesion area, and induced significant decreases in levels of TC (SMD = -2.67, 95% CI -3.68 to -1.67, $P < 0.00001$), TG (SMD = -2.43, 95% CI -3.39 to -1.47, $P < 0.00001$), and LDL-C (SMD = -2.87, 95% CI -4.16 to -1.58, $P < 0.00001$), as well as increasing HDL-C (SMD = 2.04, 95% CI 1.05 to 3.03, $P = 0.001$). TMP also significantly modulated plasma inflammatory responses and biological signals associated with atherosclerosis. In subgroup analysis, the groups of high-dose TMP (≥ 50 mg/kg) showed better results than those of the control group. No difference between various durations of treatment groups or various assessing location groups.

Conclusion: TMP exerts anti-atherosclerosis functions in an animal model of AS mediated by anti-inflammatory action, antioxidant action, ameliorating lipid metabolism disorder, protection of endothelial function, antiplatelet activity, reducing the proliferation and migration of smooth muscle cells, inhibition of angiogenesis, antiplatelet aggregation. Due to the limitations of the quantity and quality of current studies, the above conclusions need to be verified by more high-quality studies.

Li, S., et al. (2022). The role and mechanism of tetramethylpyrazine for atherosclerosis in animal models: A systematic review and meta-analysis. *PloS one*, 17(5), e0267968. doi.org/10.1371/journal.pone.0267968

Objectives: The role and mechanism of tetramethylpyrazine (TMP) in cardio-cerebrovascular diseases (CCVDs), as well as the research of its new formulations are reviewed, which provides a new strategy for the clinical application of TMP.

Methods: We searched the databases including PubMed, Web of Science, Google Scholar and CNKI for relevant literature from 1991 to 2021 by searching for the keywords "TMP", "ligustrazine", "cardiovascular disease" and "nanoformulation". The inclusion criteria are as follows: (1) the literature is an experimental article, (2) the article studies cardiovascular and cerebrovascular-related diseases and (3) the article also includes the pharmacy research of TMP. A total of 160 articles were screened.

Key findings: TMP has various pharmacological effects in the treatment of many CCVDs, such as atherosclerosis, myocardium, cerebral ischemia, reperfusion injury and hypertension. Its protective effects are mainly related to its anti-platelet activity, protection of endothelial cells, and anti-inflammation, anti-oxidant and anti-apoptotic effects. In addition to pharmacological activity studies, the information of the new formulations is also significant for the further development and utilization of TMP.

Conclusions: Above all, TMP can protect cardio-cerebro vessels, and preparing new formulations can improve its bioavailability, indicating that TMP has broad prospects in the treatment of CCVDs.

Keywords: atherosclerosis; bioavailability; cardio-cerebrovascular diseases; hypertension; ischemia reperfusion; tetramethylpyrazine.

Zhang, Y., et al.. (2022). Research progress on the pharmacy of tetramethylpyrazine and its pharmacological activity in cardiovascular and cerebrovascular diseases.

In this study, we identified tetramethylpyrazine (TMP) as a promising drug candidate for cardiac dysfunction caused by simulated high-altitude exposure. By utilizing hypobaric chambers to simulate high-altitude environments, we found that TMP improved cardiac function, alleviated cardiac hypertrophy, and reduced myocardial injury in hypobaric hypoxic mice. RNA sequencing showed that TMP also upregulated heart-contraction-related genes that were suppressed by hypobaric hypoxia exposure. Mechanistically, TMP inhibited hypobaric hypoxia-induced cardiac Ca^{2+} /calmodulin-dependent kinase II (CaMKII) activation and exerted cardioprotective effects by inhibiting CaMKII. Our data suggest that TMP application may be a promising approach for treating high-altitude-induced cardiac dysfunction, and they highlight the crucial role of CaMKII in hypobaric hypoxia-induced cardiac pathophysiology. As taken from Zhang P et. Al.2024. Int J Mol Sci.

Experimentally, TMP can significantly reduce the hemorheological indexes of rats with myocardial ischemia, and display a protective effect on MIRI (Liu et al., 2011). Coronary endothelial injury begins at the early stage of reperfusion and gradually intensifies. Xu et al. demonstrated that TMP had a protective effect on the endothelium and could promote the relaxation of the rat aortic ring at both the organ and cellular levels (Xu et al., 2014). Furthermore, TMP can be used to treat ischemic heart disease because of its inhibitory role in coronary vasoconstriction (Liu, Lin, Chen, Huang, & Lin, 2005). Myocardial cell membrane defect is an early feature of irreversible injury of ischemic cardiomyocytes. (...) It was found that TMP significantly reduced the levels of CK and LDH after I/R injury, indicating that TMP can protect myocardial cell membrane (Zhou, Hu, Deng, Deng, & Li, 2004; Zuo et al., 2019). ST segment elevation is the most direct method to diagnose myocardial ischemia. A study showed that TMP significantly reduced ST segment elevation in acute myocardial infarction rats, indicating that TMP positively affects cardiac activity (Yang et al., 2019). In clinical practice, myocardial infarction is closely related to myocardial hypertrophy. Ji et al. induced primary neonatal rat cardiomyocytes with isoproterenol to construct a model of myocardial hypertrophy. By using this model, they demonstrated that TMP could prevent myocardial hypertrophy (Ji, Song, Qian, Yu, & Zhu, 2014). Extensive fibrosis following cardiomyocyte apoptosis causes myocardial remodeling and malignant arrhythmias, which can be prevented by pretreatment with TMP (Wang et al., 2021). Moreover, the protective effect of TMP on ischemic heart is, at least in part, attributed to its anti-inflammatory effect. In addition, the role of TMP in suppression of ventricular arrhythmias caused by myocardial ischemia is believed to inhibit the migration and respiratory burst of activated neutrophils (Chen, Hsiao, Hwang, Cheng, & Lee, 2006). As taken from Qi M et. al. 2024. Pharmacol Ther.

6.3. Nervous system

“To evaluate the neuroprotective effect of tetramethylpyrazine (TMP) against focal cerebral ischemic injury in rats with diffusion-weighted magnetic resonance imaging (DWMRI). Rat models of focal cerebral ischemic injury were established in 16 male SD rats. They were randomly divided into the TMP group and the control group, eight in each group, and pretreated with TMP and normal saline respectively before modeling. Change of infarcted cerebral focus was observed with

DWMRI at 1, 2, 6, 12 and 24 hrs after infarction, and the infarction volume (IV) at 24 hrs after modeling was estimated by triphenyltetrazolium chloride (TTC) stain. The IV in all time points observed in the TMP group with DWMRI was significantly smaller than that in the control group ($P < 0.01$). Compared with that at 1 hr after infarction, in the control group at 2, 6, 12 and 24 hrs after modeling, the IV enlarged by 13.3%, 29.7%, 50.3% and 57.3% respectively, while that in the TMP group 9.9%, 21.3%, 37.1% and 40.5% respectively. The cerebral IV estimated by TTC stain 24 hrs after modeling was larger than that estimated by DWMRI. TMP pretreatment before modeling was effective in protecting brain against cerebral ischemic damage in rats. DWMRI dynamic scanning observation has important significance in observing the cerebral ischemic developing process and evaluating the effectiveness of brain protective measures." As taken from Hu S et al. *Zhongguo Zhong Xi Yi Jie He Za Zhi*. 2005. 25(2), 134-7. PubMed, 2010

"Tetramethylpyrazine (TMP), which is widely used in the treatment of ischemic stroke by Chinese herbalists, is one of the most important active ingredients of the traditional Chinese herbal medicine, *Ligusticum wallichii* Franchet (Chung Xiong). However, the mechanism by which TMP protects the brain is still not clear. We examined neuroprotective effects of TMP after transient focal cerebral ischemia using common carotid artery and middle cerebral artery occlusion model in rats and evaluated the involvement of anti-inflammation. TMP administrated intraperitoneally significantly protected the brain against ischemic insult as evidenced by the reduction in infarction volume, preservation of neurons, and decrease in brain edema. TMP markedly reduced cerebral ischemia/reperfusion-induced inflammatory cell activation and proinflammatory mediator production. Moreover, TMP suppressed lipopolysaccharide/interferon-gamma-induced inflammation and prostaglandin E(2) production in cultured glial cells. Our findings suggest that one of neuroprotective effects of TMP against ischemic brain injury might involve its anti-inflammatory potential." As taken from Liao SL et al. *Neurosci. Lett*. 2004. 372(1-2), 40-5. PubMed, 2010

"Lower limb paralysis occurs in 11% of patients after surgical procedure of thoracic or thoracoabdominal aneurysms and is an unpredictable and distressful complication. The aim of this study was to investigate the effects of tetramethylpyrazine (TMP), an intravenous drug made from traditional Chinese herbs, on the neurologic outcome and histopathology after transient spinal cord ischemia in rabbits. Forty-five male New Zealand white rabbits were anesthetized with isoflurane and spinal cord ischemia was induced for 20 min by infrarenal aortic occlusion. Animals were randomly allocated to one of five groups ($n = 8$ each). Group C received no pharmacologic intervention. Group P received intravenous infusion of 30 mg x kg(-1) TMP within 30 min before aortic occlusion. Group T1, Group T2 and Group T3 received intravenous infusion of 15, 30 and 60 mg x kg(-1) TMP respectively within 30 min after reperfusion. In the sham group ($n = 5$), the animals underwent the same procedures as the control group except infrarenal aortic unocclusion. Neurologic status was scored by using the Tarlov criteria (in which 4 is normal and 0 is paraplegia) at 4 h, 8 h, 12 h, 24 h, and 48 h after reperfusion. All animals were sacrificed at 48 h after reperfusion and the spinal cords (L5) were removed immediately for histopathologic study. All animals in the control group became paraplegic. Neurologic status and histopathology (48 h) in the Groups P, T2 and T3 were significantly better than those in the control group ($P < 0.05$). There was a strong correlation between the final neurologic scores and the number of normal neurons in the anterior spinal cord ($r = 0.776$, $P < 0.01$). Tetramethylpyrazine significantly reduces neurologic injury related to spinal cord ischemia and reperfusion after aortic occlusion within a certain range of dose." As taken from Chen S et al. *BMC Neurol*. 2002. 2, 1. PubMed, 2010

"The effect of tetramethylpyrazine on ischemic neuronal damage was studied in gerbil hippocampus in the terms of histopathological change and cerebral tissue lipid peroxides. Fifteen-five Mongolian gerbils were randomly assigned to one of three groups: sham-operated as control, subjected to 12 min global cerebral ischemia followed by 7 day spontaneous circulatory reperfusion, in which animals were treated with either ip. physiological saline or 60 mg/kg of tetramethylpyrazine 30 min before ischemia and daily thereafter for 7 days. The number of survival pyramidal neurons in the CA1 was counted: 263 +/- 8 (cell/mm) in the sham-operated group, 20 +/- 6 in the ischemia group, and 189 +/- 56 in the group treated with tetramethylpyrazine. Changes in

lipid peroxides, expressed as malondialdehyde (MDA), was 134.5 +/- 5.0 nmol/g tissue in the sham-operated group, 193.5 +/- 5.1 in the ischemia group, and 137.6 +/- 10.8 in the group treated with tetramethylpyrazine. These results indicate that tetramethylpyrazine has a protective effect on the ischemic neuronal damage in hippocampus. Free radicals and free calcium may play an important role in pyramidal neuron necrosis in hippocampus following cerebral ischemia." As taken from Lou XX et al. No To Shinkei. 1994. 46(9), 841-6. PubMed, 2010

"Tetramethylpyrazine (TMP) is the major component extracted from the Chinese herb, Chuanxiong. This study focuses on the protective effect of tetramethylpyrazine in kainate-induced excitotoxicity in rat hippocampus. Primary neuronal cultures raised from cells isolated from the hippocampi of 7-day old rats were treated with kainate (75-450 microM) for 12, 24, and 48 h. Our results revealed that kainate induced neuronal damage in a dose- and time-dependent manner, reaching maximal damage at 150 microM and 24 h and persisted for higher doses and 48 h. In addition, 1 h of kainate (150 microM) treatment led to significant generation of free radicals and reduction of mitochondrial membrane potential (MMP) which persisted for > or = 4 h on continued exposure. Ten minutes pretreatment with 1 or 5 microM tetramethylpyrazine dose dependently and significantly attenuated the kainate-induced damage. Taken together, the results suggest that multiple mechanisms including protection of mitochondria, decrease in free radical generation and scavenging of free radicals might be involved in TMP's protection against kainate induced cell toxicity." As taken from Shih YH et al. Neuroreport. 2002. 13(4), 515-9. PubMed, 2010

"traditional Chinese medicine, Ligusticum wallichii Franchat (Chuan Xiong) and its active ingredient tetramethylpyrazine (TMP) have been used to treat cardiovascular diseases and to relieve various neurological symptoms such as ischemic deficits. However, scientific evidence related to their effectiveness or precise modes of neuroprotective action is largely unclear. In the current study, we elicited the neuroprotective mechanisms of TMP after focal cerebral ischemic/reperfusion (I/R) by common carotid arteries and middle cerebral artery occlusion model in rats. TMP was administrated 60 min before occlusion via intraperitoneal injection. TMP concentration-dependently exhibited significant neuroprotective effect against ischemic deficits by reduction of behavioral disturbance. Neuronal loss and brain infarction in the ischemic side of rats were markedly lowered by treatment with TMP. Cerebral I/R-induced internucleosomal DNA fragmentation, caspase-8, caspase-9, and caspase-3 activation, and cytochrome c release were reduced by TMP treatment. Western blot analysis revealed the down-regulation of Bcl-2 and Bcl-xL and the up-regulation of Bax and Bad by cerebral I/R insult. Among them, only the alteration in Bcl-xL expression was reversed by TMP treatment. Moreover, the activation of microglia and/or recruitment of inflammatory cells within the ischemic side and the consequent production of monocyte chemoattractant protein 1 (MCP-1) were suppressed by TMP pre-treatment. Our findings suggest that TMP might provide neuroprotection against ischemic brain injury, in part, through suppression of inflammatory reaction, reduction of neuronal apoptosis, and prevention of neuronal loss." As taken from Kao TK et al. Neurochem. Int. 2006. 48(3), 166-76. PubMed, 2010

"To evaluate the effect of tetramethylpyrazine (TMP) injection on retinal damage induced by N-methyl-N-nitrosourea (MNU) in rats and on nuclear factor-kappa B (NF-kappaB) family members. Female Sprague-Dawley (SD) rats were randomly divided into groups: (i), control group; (ii), model group; and (iii), TMP-injection groups, in which the rats were subdivided into 40 mg/kg, 80 mg/kg and 160 mg/kg groups. Drugs were injected ip into 47-day-old SD rats once a day. At 50 days of age, all rats in the model group and drug groups also received a single ip injection of 60 mg/kg MNU. Rats in group 1 received ip injection of physiological saline. All rats were killed at different times after MNU or physiological saline treatment. The apoptotic index of photoreceptor cells was calculated by TUNEL labeling; retinal damage was evaluated based on retinal thickness and the expression of NF-kappaB family members was detected by Western blot. TMP injections, in a dose-dependent manner, suppressed photoreceptor cell apoptosis and decreased its loss in the peripheral retina. As compared with the MNU-treated group, TMP injection at a dose of 160 mg/kg also time-dependently upregulated the NF-kappaB/p65 protein level in the nucleus and downregulated the I kappa B alpha protein level in the cytoplasm. However, no protective effect of

TMP injection on MNU-induced central retinal damage was found. TMP injection partially protects against MNU-induced retinal damage by upregulating the nuclear translocation of p65 to inhibit photoreceptor cells apoptosis." As taken from Jang JN et al. *Acta Pharmacol. Sin.* 2005. 26(7), 887-92. PubMed, 2010

"Tetramethylpyrazine (TMP) is one of the alkaloids contained in Ligustrazine which has been used in traditional Chinese medicine as an analgesic for injury and dysmenorrhea. ATP can elicit the sensation of pain. This study observed the effects of TMP on ATP-activated current (IATP) in rat DRG neurons. TMP (0.1-1 mM) concentration-dependently inhibited ATP (100 microM)-activated current in rat DRG neurons. The inhibitory time of ATP (100 microM)-activated current appeared at 15 s after preapplication of TMP and reached its peak at about 45 s. The dose-response curves for IATP in the absence and presence of 1 mM TMP showed that TMP (1 mM) shifted the concentration-response curve of IATP downward markedly and the two EC₅₀ values were very close (75 vs. 82 microM), while the threshold value remained unchanged. Therefore, the inhibitory effect of TMP on IATP may be noncompetitive. TMP did not alter the reversal potential (0 mV) of ATP-activated current, indicating that the site of TMP action is on or near the exterior surface of channel protein and not within the channel pore. Externally applied TMP (1 mM) increases the inhibitory effect of chelerythrine (PKC inhibitor) contained in pipette solution on IATP. The site of TMP action may be the binding of TMP to an allosteric site on the large extracellular region of ATP receptor-ion channel complex (P2X receptors) or PKC site of the N-terminus of P2X receptors. The mechanism of TMP action may be the allosteric regulation via acting on the large extracellular region of ATP receptor-ion channel complex (P2X receptors) and promoting the phosphorylation of PKC site of the N-terminus of P2X receptors." As taken from Liang SD et al. *Brain Res.* 2005. 1040(1-2), 92-7. PubMed, 2010

"To explore the protective effect of tetramethylpyrazine (TMP) on the learning and memory function in D-galactose (D-gal)-lesioned mice. C57BL/6 mice were injected (s.c.) 2% D-gal for 40 days (100 mg x kg(-1) x d(-1)). Normal saline, TMP, and Huperzine A were respectively given by intragastric administration in different groups from the third week. Learning and memory ability was tested with Morris water maze for 5 days at the sixth week. After completion of behavioral test, the mice were sacrificed by decapitation. The brain was rapidly removed, and the cortex and hippocampus were separated. The superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in the cortex were determined. At the same time, the activity of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), the binding sites (B_{max}) and the affinity (K_D) of M-cholinergic receptor in the cortex, and B_{max} and K_D of N-methyl-D-aspartate (NMDA) receptor in the hippocampus were determined. In this model group, (1) The deficit of learning and memory ability, (2) elevated MDA content and lowered SOD activity, (3) decreased AChE activity and M-cholinergic receptor binding sites in the cortex, and (4) lowered NMDA receptor binding sites were observed in the hippocampus, as compared with the normal control. TMP could markedly (1) attenuate cognitive dysfunction, (2) lower MDA content and elevate SOD activity, (3) increase the activity of ChAT and AChE, and M-cholinergic receptor binding sites in the cortex in the mice treated with D-gal. NMDA receptor binding sites were also increased in the hippocampus in the treated mice. TMP can significantly strengthen antioxidative function, improve central cholinergic system function, protect NMDA receptor activity, and thus enhance the learning and memory ability in D-gal-lesioned mice." As taken from Zhang C et al. *Chin. Med. Sci. J.* 2004. 19(3), 180-4. PubMed, 2010

"To evaluate the effects of tetramethylpyrazine (TMP) on retinal neovascularization (NV) and neuroprotection in an oxygen-induced retinopathy (OIR) model. Neonatal C57BL/6J mice were subjected to 75% oxygen from postnatal day 7 (P7) to P12 and then returned to room air. TMP (200 mg/kg) or normal saline was given daily from P12 to P17. Immunostaining, HE staining, TUNEL assay, and RT-PCR were used to assess the effects of TMP on retinal neurovascular repair. TMP effectively prevented pathologic NV and accelerated physiologic revascularization by enhancing the formation of endothelial tip cells at the edges of the repairing capillary networks and preserving the astrocytic template in the avascular retina. TMP also prevented morphologic changes and significantly decreased TUNEL-positive cells in the avascular retina by rescuing neurons such as

amacrine, rod bipolar, horizontal, and Müller cells. In TMP-treated mice retinas, there was a less obvious loss of amacrine cell bodies and their distinct bands; the number of both rod bipolar and horizontal cell bodies, as well as the density of their dendrites in the outer plexiform layer, was greater than that in OIR control mice. TMP not only decreased the loss of alignment of Müller cell bodies and distortion of processes but reduced the reactive expression of GFAP in Müller cells. Furthermore, HIF-1 α and VEGF mRNA expression were downregulated in TMP-treated mice retinas. TMP improved neurovascular recovery by preventing NV and protecting retinal astroglia cells and neurons from ischemia-induced cell death partially due to its downregulation of HIF-1 α and VEGF mRNA expression. TMP prevents retinal neovascularization and imparts neuroprotection in an oxygen-induced retinopathy model.” As taken from Liang X et al. *Invest. Ophthalmol. Vis. Sci.* 2012. 53(4), 2157-69. doi: 10.1167/iov.11-9315. PubMed 2013

“Tetramethylpyrazine (TMP), one of the active ingredients of the Chinese herb *Lingusticum Wallichii* Franchet (Chuan Xiong), plays an important role in neuroprotection. However, the protective effect of TMP on the medulla oblongata, the most important region of the brain for cardiovascular and respiratory control, during chronic hypoxia remains unclear. In this study, we examined the neuroprotective effect of TMP on the medulla oblongata after chronic hypoxic injury in rats. Male Sprague-Dawley rats were randomly divided into four groups: control group, TMP group, chronic hypoxia group, and chronic hypoxia+TMP group. Rats were exposed to hypoxia (10% (v/v) O₂) or normoxia for 6 h daily for 14 days. TMP (80 mg/kg) or vehicle (saline) was injected intraperitoneally 30 min before experimentation. Loss of neurons in the pre-Bötzinger complex, the nucleus ambiguus, the nucleus tractus solitarius, the hypoglossal nucleus and the facial nucleus were evaluated by Nissl staining. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were measured, and apoptosis was monitored using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. The level of Bcl-2 mRNA and Bax mRNA was quantitatively measured by RT-PCR analysis. TMP protected Nissl bodies of neurons from injury in all nuclei observed, and reduced the loss of neurons in the nucleus ambiguus, the nucleus tractus solitarius, and the hypoglossal nucleus in rats subjected to chronic hypoxia. TMP upregulated SOD activity and inhibited the increase in MDA content in the medulla oblongata of hypoxic rats. In addition, TMP decreased the rate of apoptosis index (the percentage of apoptotic cells against the total number of cells) in all medullary structures examined, excepting the nucleus ambiguus and inhibited the decrease in Bcl-2 mRNA levels in the medulla oblongata following hypoxia. Our findings indicate that TMP may protect the medullary structures that are involved in cardiovascular and respiratory control from injury induced by chronic hypoxia in rats via its anti-oxidant and anti-apoptotic effects.” As taken from Ding Y et al. 2013. *Auton. Neurosci.* 173(1–2), 45–52. PubMed, 2013

“Tetramethylpyrazine (TMP) has been used to treat ischemic stroke. However, scientific evidence related to its effectiveness or precise modes of neuroprotective action is largely unclear. This study provides evidence of an alternative target for TMP and sheds light on the mechanism of its physiological benefits. We report a global inhibitory effect of TMP on intracerebral cellular inflammatory response in a rat model of permanent cerebral ischemia. TMP exhibited a neuroprotective effect against ischemic deficits by reduction of behavioral disturbance, brain infarction, and edema. The results of immunohistochemistry, enzymatic assay, Western blot, real-time reverse transcriptase-polymerase chain reaction (RT-PCR), and flow cytometric analysis revealed that TMP reduced the percentages of activated macrophages/microglia and infiltrative lymphocytes, neutrophils, and macrophages and pro-inflammatory cytokine expression after cerebral ischemia. In parallel with these immunosuppressive phenomena, TMP also attenuated the activities of ischemia-induced inflammation-associated signaling molecules and transcription factors. Another finding in this study was that the anti-inflammatory and neuroprotective effects of TMP were accompanied by a further elevated expression of NF-E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in ipsilateral neurons and macrophages/microglia after cerebral ischemia. Taken together, our results suggest that both the promotion of endogenous defense capacity and the attenuation of the extent and composition percentage of the major cellular

inflammatory responses via targeting of macrophages/microglia by elevating Nrf2/HO-1 expression might actively contribute to TMP-mediated neuroprotection against cerebral ischemia.” As taken from Kao TK et al. 2013. *Exp. Neurol.* 247, 188–201. PubMed, 2013

“OBJECTIVE: WE STUDIED THE EFFECTS OF TETRAMETHYLPYRAZINE(TMP) ON THE FEVER, INCREASED PLASMA LEVELS OF TUMOR NECROSIS FACTOR- α (TNF- α) and increased hypothalamic levels of glutamate, hydroxyl radicals and prostaglandin-E2 (PGE2) induced by lipopolysaccharide (LPS). MATERIALS AND METHODS: The microdialysis probes were stereotactically and chronically implanted into the hypothalamus of rabbit brain for determining extracellular levels of glutamate, hydroxyl radicals, and PGE2. In addition, both the body core temperature and plasma levels of TNF- α were measured. RESULTS: All the body core temperature, plasma levels of TNF- α , and hypothalamic levels of glutamate, hydroxyl radicals, and PGE2 were up-graded by an intravenous dose of LPS (2 μ g/kg). Pretreatment with intravenous TMP (10-40 mg/kg) or intracerebroventricular TMP (130 μ g in 20 μ l per animal) 1 h before LPS administration significantly attenuated the LPS-induced fever as well as the increased hypothalamic levels of glutamate, hydroxyl radicals, and PGE2. LPS-induced fever could also be attenuated by intravenous or intracerebroventricular TMP 1 h after LPS injection. CONCLUSION: TMP preconditioning may cause its antipyretic action by reducing plasma levels of TNF- α as well as hypothalamic levels of glutamate, hydroxyl radicals, and PGE2 in rabbits.” As taken from Chang CH et al. 2013. *Inflamm. Res.* 62(5), 527–35. PubMed, 2013

“The survival in patients with malignant gliomas still remains limited and novel treatment strategies are urgently needed. Tetramethylpyrazine (TMP) extracted from the Chinese herb Chuanxiong, has been suggested to have a therapeutic potential towards glioma primarily through its neural protection activity. However, the exact mechanisms correlating TMP's antitumor function and neural protection have not been yet elucidated. Thus, this study aimed to investigate TMP's molecular target in tumor inhibition and neural protection. The primary cultured cerebral neurocytes were treated with 100 μ M TMP for 14 days in vitro. We found TMP can effectively promote neurons survival, compared to controls. TMP effectively inhibits H₂O₂-induced rise of [Ca(2+)]_i and glutamate releasing in cerebral neurocytes, compared to controls. In addition, we verify previous results that TMP significantly decreases the migration and proliferation of C6 glioma cells. Using glioma-neuronal co-culturing system, we further confirm TMP bioactivity in inhibition of glioma cells and protection of cerebral neurocytes. More importantly, our study demonstrates that the expression of chemokine receptor, CXCR4, which plays a key role in tumor development and various neurodegenerative diseases, is significantly decreased in both cerebral neurocytes and C6 glioma cells with TMP treatment, cultured alone or co-cultured. Compared with CXCR4 antagonist, AMD3100, TMP is more effective on glioma inhibition and neural protection. Glutamate concentration in medium of co-culturing system was lower after treatment with 100 μ M TMP. Therefore, our findings suggest that TMP-mediated suppression of C6 gliomas and neural protection involves inhibition of CXCR4 expression. Thus, this study provides new insights into TMP's therapeutic potential in the treatment of malignant gliomas.” As taken from Chen Z et al. 2013. *Cancer Lett.* 336(2), 281–9. PubMed, 2013

“Secondary mechanisms, including inflammation and microglia activation, serve as targets for the development and application of pharmacological strategies in the management of spinal cord injury (SCI). Tetramethylpyrazine (TMP), an active ingredient of *Ligusticum wallichii* (chuanxiong), has shown anti-inflammatory and neuroprotective effects against SCI. However, it remains uncertain whether the inflammation-suppressive effects of TMP play a modulatory role over microglia activation in SCI. The present study investigated the effects of TMP on microglia activation and pro-inflammatory cytokines in spinal cord compression injury in mice. For a real-time PCR measurement of pro-inflammatory cytokines, SCI was induced in mice by the clip compression method (30 g force, 1 min) and TMP (15 or 30 mg/kg, i.p.) was administered once, 30 minutes before the SCI induction. For immunohistochemistry, TMP (30 mg/kg, i.p.) treatment was given three times during the first 48 hours after the SCI. 30 mg/kg of TMP treatment reduced the up-

regulation of TNF- α , IL-1 β and COX-2 mRNA in the spinal tissue at four hours after the SCI induction. TMP also significantly attenuated microglia activation and neutrophil infiltration at 48 hours after the SCI induction. In addition, iNOS expression in the spinal tissue was attenuated with TMP treatment. These results suggest that TMP plays a modulatory role in microglia activation and may protect the spinal cord from or potentially delay secondary spinal cord injury.” As taken from Shin JW et al. 2013. Am. J. Chin. Med. 41(6), 1361–76. PubMed, 2013

“...A liquid chromatography-electrochemical detector method has been established and validated for the determination of dopamine in rat brain dialysate. The results indicate that ligustrazine administration by subcutaneous injection significantly increased dopamine release in rat medial prefrontal cortex, nucleus accumbens and hippocampus in a dose-related manner.” As taken from Lu YF. 2013. Yao Xue Xue Bao 48(6), 906–10. PubMed, 2013

“Ligustrazine, one of the major effective components of the Chinese traditional medicinal herb Ligusticum Chuanxiong Hort, has been reported plenty of biological activities, such as protect cardiovascular and cerebrovascular, neuroprotection and anti-tumor, et al. Because of its remarkable effects, studies on structural modification of ligustrazine have attracted much attention. Ligustrazine synthetic derivatives reported in recent decades are mainly derived from four primary intermediates (TMP-COOH, TMP-OH, TMP-NH₂, HO-TMP-OH). To explore the neuroprotection activity of ligustrazine intermediates, six ligustrazine intermediates (2, 5, 8, 11, 12, 13) were synthesized and their protective effects against CoCl₂-induced neurotoxicity in differentiated PC12 cells were studied. The target compounds were prepared via different chemical methods, including oxidation, substitution, esterification and amidation without changing the structure nucleus of ligustrazine. Compared with TMP (EC₅₀ = 56.03 micromol x L⁻¹), four compounds (2, 5, 12 and 13) exhibited higher activity (EC₅₀ 70 micromol x L⁻¹). By structure-activity relationships analysis, the introduction of carboxyl, amino to the side chain of ligustrazine and appropriately increase the proportion of ligustrazine may contribute to enhance its neuroprotective activity, which provides a reference for the design, synthesis and activity screening of relevant series of ligustrazine derivatives in the future”. As taken from Li GL et al. 2014. Zhongguo Zhong Yao Za Zhi 39(14), 2679-83. PubMed, 2014

“This study aimed to evaluate the effect of ligustrazine on levels of amino acid transmitters in the extracellular fluid of striatum following cerebral ischemia/reperfusion (I/R) in male Sprague-Dawley rats. A microdialysis cannula guide was implanted into the right striatum. After recovery, animals underwent a sham operation or middle cerebral artery occlusion (MCAO). Those that developed cerebral ischemia after MCAO were randomized to receive propylene glycol salt water and ligustrazine respectively. Striatal fluid samples were collected from all animals at 15-min intervals after treatment and were subjected to HPLC analysis of aspartic acid, glutamic acid, taurine, and γ -amino butyric acid. Upon the last sample collection, animals were sacrificed and brain tissue specimens were collected for triphenyltetrazolium chloride staining and NeuN staining. Compared with the sham operation, MCAO induced significant neurological deficits and increased striatal concentrations of the four neurotransmitters assessed in a time-dependent manner (P<0.01). Ligustrazine effectively attenuated the detrimental effects of MCAO on the brain. These observations suggest that ligustrazine as a novel cerebral infarction-protective agent may have potential clinical implications for I/R-related brain damage”. As taken from Han J et al. 2014. J. Asian Nat. Prod. Res. 16(11), 1060-7. PubMed, 2015

“Recent studies showed that pathology of alcoholic encephalopathy was associated with cerebral vascular damage. TMP (tetramethyl- pyrazine) is widely used in the treatment of cerebrovascular diseases, however, it has not been reported whether TMP can relieve alcohol-induced cerebral vascular damages. The study was performed to investigate the learning and memory, cerebrovascular pathological changes and the expressions of vascular endothelial growth factor (VEGF) and serum levels of endothelin-1 (ET-1) in the rat model of chronic alcoholic encephalopathy, and explore the effects of TMP intervention on alcoholic encephalopathy. In the present study, the rat model of chronic alcoholic encephalopathy was established by the gavage

administration of alcohol; the learning and memory ability was tested by Morris water maze; the expression of VEGF was measured by RT-PCR and Western blot; and the serum levels of ET-1 was measured by radioimmunoassay. We found that alcohol intoxication impaired learning and memory, induced VEGF overexpression and increased ET-1 concentrations. TMP intervention improved learning abilities, increased the VEGF expression and reduced ET-1 level. These results indicate that TMP exhibits therapeutic effects on chronic alcoholic encephalopathy.” As taken from Li H et al. 2015. *Biomed. Environ. Sci.* 28(9), 691-5. PubMed, 2016

“Hypoxia-mediated neurotoxicity contributes to various neurodegenerative disorders, including Alzheimer's disease and multiple sclerosis. Tetramethylpyrazine (TMP), a major bioactive component purified from *Ligusticum wallichii* Franchat, exhibited potent neuroprotective effect. However, the mechanism of TMP-exerted neuroprotective effect against hypoxia was not clear. In the study, we investigated the mechanism of the neuroprotective effect of TMP against hypoxia induced by CoCl₂ in vitro and in vivo. The results showed that TMP could protect against CoCl₂ -induced neurotoxicity in PC12 cells and in rats, as evidenced by enhancement of cell viability in PC12 cells and improvement of learning and memory ability in rats treated with CoCl₂. TMP could inhibit mitochondrial dysfunction, mitochondrial apoptotic molecular events, and thus apoptosis induced by CoCl₂. TMP inhibited CoCl₂ -increased reactive oxygen species (ROS) level, which may contribute to hypoxia-related neurotoxicity induced by CoCl₂. The antioxidant and neuroprotective activities of TMP involved two pathways: one was the enhancement of nuclear factor erythroid 2-related factor 2 (Nrf2)/catalytic subunit of γ -glutamylcysteine ligase-mediated regulation of GSH and the other was the inhibition of hypoxia-inducible factor 1 α /NADPH oxidase 2 (NOX2)-mediated ROS generation. These two pathways contributed to improvement of oxidative stress and thus the amelioration of apoptosis under hypoxic conditions. These results have appointed a new path toward the understanding of pathogenesis and TMP-related therapy of hypoxia-related neurodegenerative diseases. We proposed two cascades for tetramethylpyrazine-exhibited protective effects against CoCl₂ -induced neurotoxicity: One is enhancement of nuclear factor erythroid 2-related factor 2-catalytic subunit of γ -glutamylcysteine ligase-mediated regulation of glutathione and the other was the inhibition of hypoxia-inducible factor 1 α -NADPH oxidase-2-mediated ROS generation. We think these findings should provide a new understanding of pathogenesis and tetramethylpyrazine-related therapy of hypoxia-related neurodegenerative diseases.” As taken from Guan D et al. 2015. *J. Neurochem.* 134(3), 551-65. PubMed, 2016

“Experimental studies have demonstrated the beneficial effects of tetramethylpyrazine (TMP) against ischemic stroke and highlighted its crucial role in anti-inflammatory activity. This study provides evidence of an alternative target for TMP and sheds light on the mechanism of its anti-inflammatory action against ischemic brain injury. We report a global inhibitory effect of TMP on inflammatory cell intracerebral activation and infiltration in a rat model of permanent cerebral ischemia. The results of immunohistochemistry, enzymatic assay, flow cytometric analysis, and cytological analysis revealed that intraperitoneal TMP administration reduced neuronal loss, macrophage/microglia activation, brain parenchyma infiltrative neutrophils, and circulating neutrophils after cerebral ischemia. Biochemical studies of cultured neutrophils further demonstrated that TMP attenuated neutrophil migration, endothelium adhesion, spontaneous nitric oxide (NO) production, and stimuli-activated NO production after cerebral ischemia. In parallel with these anti-neutrophil phenomena, TMP also attenuated the activities of ischemia-induced inflammation-associated signaling molecules, including plasma high-mobility group box-1 protein (HMGB1) and neutrophil toll-like receptor-4 (TLR4), Akt, extracellular signal-regulated kinase (ERK), and inducible nitric oxide synthase. Another finding in this study was that the anti-neutrophil effect of TMP was accompanied by a further elevated expression of NF-E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) in neutrophils after cerebral ischemia. Taken together, our results suggest that both the promotion of endogenous anti-inflammatory defense capacity and the attenuation of pro-inflammatory responses via targeting of circulating neutrophils by elevating Nrf2/HO-1 expression and inhibiting HMGB1/TLR4, Akt, and ERK signaling might actively

contribute to TMP-mediated neuroprotection against cerebral ischemia.” As taken from Chang CY et al. 2015. *Biochem. Biophys. Res. Commun.* 463(3), 421-7. PubMed, 2016

“AIMS: Neurodegenerative diseases are the leading cause of morbidity and mortality worldwide. Several studies have shown that tetramethylpyrazine (TMP) is an effective therapy for neurodegenerative diseases and that it acts by inhibiting the activation of microglial cells in response to inflammatory stimuli. However, the molecular mechanisms underlying the action of TMP remain unknown. MAIN METHODS: Proteomic analysis was used to generate novel insights into the mechanism by which TMP inhibits microglial activation, and western blotting was used to validate candidate proteins. KEY FINDINGS: To identify candidate proteins affected by TMP in lipopolysaccharide-activated microglia, we performed proteomic analysis using iTRAQ labelling coupled with LC TRIPLE-TOF, and we identified 5187 unique proteins. Among these, 266 proteins were differentially expressed and considered putative candidate proteins. Protein annotation revealed that the differentially expressed proteins, such as inducible nitric oxide synthase (iNOS) and ERO1-like protein (ERO1L), might be involved in reducing cellular oxidation in response to stress. Ingenuity pathway analysis revealed that the differentially expressed proteins were involved in a variety of signalling pathways, including liver X receptor/retinoid X receptor (LXR/RXR) activation and the production of nitric oxide and reactive oxygen species in macrophages. Furthermore, one of the differentially expressed protein candidates detected by iTRAQ, iNOS, was confirmed by western blotting. SIGNIFICANCE: Our data suggest that iTRAQ technology is an effective tool to study the mechanism by which TMP inhibits activated microglia. TMP decreased the expression of LXR/RXR-mediated iNOS, which reduced microglial activation in response to inflammatory stimuli.” As taken from Pu QH et al. 2015. *Life Sci.* 121, 46-56. PubMed, 2016

“Tetramethylpyrazine (TMP), one of the active ingredients of the Chinese herb *Lingusticum Wallichii* (Chuan Xiong) has been proved to protect the medulla oblongata from chronic hypoxia injury. However, the underlying mechanism remains unclear. The purpose of this study was to determine whether the protective effects of TMP are associated with the heme oxygenase-1 (HO-1) dependant pathway in adult rats. The morphological changes of neurons in the hypoglossal nucleus (12N), the nucleus ambiguus (Amb), the nucleus tractus solitarius (NTS), and the pre-Bötzinger complex (pre-BötC) were investigated by Nissl staining; the malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were measured to evaluate the anti-oxidant effect; some apoptosis parameters, Bax mRNA and Bcl-2 mRNA, were tested; and the double immunohistochemistry staining of active caspase-3/NeuN was performed. Meanwhile, the HO-1 protein expression and heme oxygenase (HO) activity were examined. Tin-protoporphyrin (SnPP), a potent inhibitor of HO, was used to further confirm the effect of HO-1. We found that TMP ameliorated the neuron loss in 12N, Amb and NTS, the decrease in SOD activity and the increase in MDA content, the decrease in Bcl-2 mRNA of medulla oblongata ($P<0.05$), and the increase in percentage of apoptotic neurons in Amb ($P<0.05$) induced by chronic hypoxia. Co-administration with SnPP abolished the beneficial effects above of TMP to some extent ($P<0.05$). Moreover, TMP significantly increased HO activity and HO-1 protein expression, which was most likely enhanced in the neurons ($P<0.05$), and co-administration of SnPP reduced these up-regulated effects ($P<0.05$). This study demonstrated that HO-1 dependant pathway may be involved in the protective action of TMP against chronic hypoxic damage on medulla oblongata in the rats.” As taken from Ding Y et al. 2015. *J. Neurol. Sci.* 361, 101-111. PubMed, 2016

“Spinal cord injury (SCI) is one of the most severe traumatic conditions, resulting in postoperative complications. Our results and other reports have shown that tetramethylpyrazine (TMP) is able to exhibit neuro-protective effects after SCI. In the current study, we aimed to examine the possible mechanism underlying the neuro-protective effect of TMP in rat model of SCI. TMP improved locomotor functions and decreased permeability of blood-spinal cord barrier in rats with SCI, as evidenced by increase of Basso-Beattie-Bresnahan scores and decrease of Evans blue leakage. In addition, TMP decreased the expression of several proinflammatory cytokines, including IL-1 β , TNF α and IL-18, reduced TUNEL-positive cells and caspase 3 and 9 activities, decreased thiobarbituric acid reactive substances content and increased glutathione level and superoxide

dismutase activity in rats. All these effects were inhibited by zinc protoporphyrin IX (ZnPP), an inhibitor of HO-1, and LY294002, an inhibitor of Akt. Moreover, TMP inhibited the decrease of mRNA expression of HO-1 which was suppressed by ZnPP and LY294002. TMP inhibited the decrease of Akt phosphorylation in rats after SCI, which was suppressed by LY294002, but not ZnPP. Furthermore, LY294002, but not ZnPP, significantly inhibited TMP-induced increase of mRNA expression of Nrf2 and DNA binding activity of Nrf2 in HO-1 promoters in rat model of SCI. The data suggested that TMP induced neuro-protective effects against injury of spinal cord through the activation of Akt/Nrf2/HO-1 signaling pathway. These results have appointed a new path toward the understanding of pathogenesis and TMP-related therapy of SCI and associated neurodegenerative diseases.” As taken from Wang C et al. 2016. *Bioorg. Med. Chem. Letters* 26(4), 1287-91. PubMed, 2016

“Tetramethylpyrazine (TMP) is an active compound extracted from the traditional Chinese medicinal herb Chuanxiong. Previously, we have shown that TMP induces human SH-SY5Y neuroblastoma cell differentiation toward the neuronal phenotype by targeting topoisomerase II β (TopoII β), a protein implicated in neural development. In the present study, we aimed to elucidate whether the transcriptional factors specificity protein 1 (Sp1) and nuclear factor Y (NF-Y), in addition to the upstream signaling pathways ERK1/2 and PI3K/Akt, are involved in modulating TopoII β expression in the neuronal differentiation process. We demonstrated that SH-SY5Y cells treated with TMP (80 μ M) terminally differentiated into neurons, characterized by increased neuronal markers, tubulin β III and microtubule associated protein 2 (MAP2), and increased neurite outgrowth, with no negative effect on cell survival. TMP also increased the expression of TopoII β , which was accompanied by increased expression of Sp1 in the differentiated neuron-like cells, whereas NF-Y protein levels remained unchanged following the differentiation progression. We also found that the phosphorylation level of Akt, but not ERK1/2, was significantly increased as a result of TMP stimulation. Furthermore, as established by chromatin immunoprecipitation (ChIP) assay, activation of the PI3K/Akt pathway increased Sp1 binding to the promoter of the TopoII β gene. Blockage of PI3K/Akt was shown to lead to subsequent inhibition of TopoII β expression and neuronal differentiation. Collectively, the results indicate that the PI3K/Akt/Sp1/TopoII β signaling pathway is necessary for TMP-induced neuronal differentiation. Our findings offer mechanistic insights into understanding the upstream regulation of TopoII β in neuronal differentiation, and suggest potential applications of TMP both in neuroscience research and clinical practice to treat relevant diseases of the nervous system.” As taken from Yan YX et al. 2015. *Eur. J. Cell Biol.* 94(12), 626-41. PubMed, 2016

“BACKGROUND: Current antidepressants are clinically effective only after several weeks of administration. Tetramethylpyrazine (TMP) is an identified component of *Ligusticum wallichii* with neuroprotective effects. Here, we investigated the antidepressant effects of TMP in mice models of depression. METHODS: Antidepressant effects of TMP were first detected in the forced swim test (FST) and tail suspension test (TST), and further assessed in the chronic social defeat stress (CSDS) model. Changes in the brain-derived neurotrophic factor (BDNF) signaling pathway and in hippocampal neurogenesis after CSDS and TMP treatment were then investigated. A tryptophan hydroxylase inhibitor and BDNF signaling inhibitors were also used to determine the mechanisms of TMP. RESULTS: TMP exhibited potent antidepressant effects in the FST and TST without affecting locomotor activity. TMP also prevented the CSDS-induced symptoms. Moreover, TMP completely restored the CSDS-induced decrease of BDNF signaling pathway and hippocampal neurogenesis. Furthermore, a blockade of the BDNF signaling pathway prevented the antidepressant effects of TMP, while TMP produced no influence on the monoaminergic system. CONCLUSIONS: In conclusion, these data provide the first evidence that TMP has antidepressant effects, and this was mediated by promoting the BDNF signaling pathway.” As taken from Jiang B et al. 2015. *Int. J. Neuropsychopharmacol.* 18, 8. PubMed, 2016

“Stem cell therapy may provide a novel therapeutic method for the replacement and regeneration of damaged neural cells in the central nervous system. However, insufficient stem cell migrating into the injured regions limits its applications. Although tetramethylpyrazine (TMP) originally isolated

from *Ligusticum walliichi* (Chuanxiong) has been widely used to treat ischemic stroke in the clinic for many years because of its role in neuroprotection, how TMP impacts the migration of neural progenitor/precursor cells (NPCs) and what is the underlying cellular and molecular mechanism remain largely unknown. Here, we found that TMP promoted NPC migration through increasing the expression and secretion of stromal cell-derived factor 1 (SDF-1), a chemokine that has been well demonstrated to direct NPC cell trafficking, in a dose-dependent fashion as analyzed by using different methods. The role of TMP in NPC migration could be inhibited by AMD 3100, a chemokine (C-X-C motif) receptor 4 (CXCR4) antagonist. Further investigation of the molecular mechanisms revealed that TMP treatment rapidly activated phosphatidylinositol 3-kinase (PI3K)/Akt, protein kinase C (PKC), and extracellular signal-regulated kinase (ERK), but not Pyk2, in NPCs. NPC migration could be blocked by using pharmacological inhibitors for these signaling pathways such as LY294002 (a PI3K inhibitor), Myr- ψ PKC (a PKC inhibitor), and an ERK1/2 inhibitor. Furthermore, TMP enhanced NPC migration toward the ischemic region in the MCAO rat model. Our findings provide mechanistic insights into the role of TMP in treating the neuropathological diseases, which suggest that TMP may be used as a potent drug for improving NPC migration in stem cell-based therapy." As taken from Kong X et al. 2016. *Mol. Neurobiol.* 53(9), 6526-6539. PubMed, 2016

"CONTEXT: Inflammation after spinal cord injury (SCI) may be responsible for further neural damages and therefore inhibition of inflammatory processes may exert a neuroprotection effect. OBJECTIVES: To assess the efficacy of some non-conventional herbal medications including sulforaphane, tanshinone IIA, and tetramethylpyrazine in reducing inflammation and compare them with a known effective anti-inflammatory agent (interleukin-10 (IL-10)). METHODS: We searched relevant articles in Ovid database, Medline (PubMed) EMBASE, Google Scholar, Cochrane, and Scopus up to June 2013. The efficacy of each treatment and study powers were compared using random effects model of meta-analysis. To our knowledge, no conflict of interest exists. RESULTS: Eighteen articles entered into the study. The meta-analysis revealed that exogenous IL-10 was more effective in comparison with the mentioned herbal extracts. The proposed pathways for each medication's effect on reducing the inflammation process are complex and many overlaps may exist. CONCLUSION: IL-10 has a strong effect in the induction of neuroprotection and neurorecovery after SCI by multiple pathways. Tetramethylpyrazine has an acceptable influence in reducing inflammation through the up-regulation of IL-10. Outcomes of sulforaphane and tanshinone IIA administration are acceptable but still weaker than IL-10" As taken from Koushki D et al. 2015. *J. Spinal Cord Med.* 38(1), 13-22. PubMed, 2016

"Tetramethylpyrazine (TMP) has been suggested to have neuroprotective effects against spinal cord injury (SCI); however, few studies have examined these effects and the corresponding mechanism. Therefore, the present study aimed to investigate the neuroprotective effect and underlying mechanism of TMP against contusive SCI. Adult male Sprague-Dawley rats were randomly divided into Sham, normal saline (NS) and TMP groups. Each group was divided into subgroups according to the time of sacrifice: 1, 3, 7, 14, 21 and 28 days post-injury. Laminectomy was performed in all groups, followed by contusive SCI establishment in the TMP and NS groups. TMP (80 mg/kg) was injected thereafter daily from 3 to 7 days post-injury in the TMP group, which was replaced by equal volume of normal saline in the NS group. The Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale was measured at different time points post-injury to appraise locomotor functional recovery. Quantitative real-time PCR and immunofluorescence were used to assess the spatio-temporal expression of peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), while western blot was adopted to detect the effect of TMP on PGC-1 α . Neural apoptotic changes and neuronal survival were evaluated using the TUNEL method and Nissl staining, respectively. TMP treatment markedly increased PGC-1 α expression, neuronal survival and BBB locomotor scores, while also reducing neural apoptosis. These results demonstrate that TMP is neuroprotective against contusive SCI, with the inhibition of neural apoptosis and increase of neuronal survival. The sustained expression of PGC-1 α may partially contribute to the TMP-mediated neuroprotective effect." As taken from Hu J et al. 2015b. *Neurochem. Res.* 40(7), 1393-401. PubMed, 2016

"The 2,3,5,6-tetramethylpyrazine (TMP) has been widely used in the treatment of ischemic stroke by Chinese doctors. Here, we report the effects of TMP on functional recovery and dendritic plasticity after ischemic stroke. A classical model of middle cerebral artery occlusion (MCAO) was established in this study. The rats were assigned into 3 groups: sham group (sham operated rats treated with saline), model group (MCAO rats treated with saline) and TMP group (MCAO rats treated with 20 mg/kg/d TMP). The neurological function test of animals was evaluated using the modified neurological severity score (mNSS) at 3 d, 7 d, and 14 d after MCAO. Animals were euthanized for immunohistochemical labeling to measure MAP-2 levels in the peri-infarct area. Golgi-Cox staining was performed to test effect of TMP on dendritic plasticity at 14 d after MCAO. TMP significantly improved neurological function at 7 d and 14 d after ischemia, increased MAP-2 level at 14 d after ischemia, and enhanced spine density of basilar dendrites. TMP failed to affect the spine density of apical dendrites and the total dendritic length. Data analyses indicate that there was significant negative correlation between mNSS and plasticity measured at 14 d after MCAO. Thus, enhanced dendritic plasticity contributes to TMP-elicited functional recovery after ischemic stroke." As taken from Lin JB et al. 2015. Evid. Based Complement. Alternat. Med. 2015, 394926. PubMed, 2016

"Ligustrazine, also known as 2,3,5,6-tetramethylpyrazine (TMP), one of the major active compounds of *Ligusticum wallichii* Franchat., has been shown to reduce neuroinflammation and protect neurons during cerebral ischemia/reperfusion injury. However, whether it reduces blood-brain barrier (BBB) permeability during ischemic stroke is unclear. The aim of the present study was to investigate the role that TMP plays in protecting the BBB integrity in ischemia/reperfusion injury and to investigate the relevant mechanisms involved. Rats received an intraperitoneal injection of 20 mg/kg TMP 15 min before the onset of ischemia, which was induced by middle cerebral artery occlusion. Infarct volume, neurological score, brain edema, BBB permeability and tight junction protein impairment were observed. The results showed that TMP reduced the neurological score and levels of brain infarction and edema. In addition, TMP significantly decreased BBB permeability and prevented the impairment of occludin and claudin-5, two tight junction protein components of the BBB, in rat brains with ischemia/reperfusion injury. In addition, the expression and activity of matrix metalloproteinases, enzymes responsible for the degradation of the extracellular matrix and tight junctions, were reduced in the rat brains by TMP treatment. These results combined suggest that TMP reduces BBB permeability as well as neuronal damage in focal cerebral ischemia/reperfusion injury in rats." As taken from Tan F et al. 2015. Exp. Ther. Med. 9(5), 1757-1762. PubMed, 2016

"The present study evaluated the ability and optimal concentration of tetramethylpyrazine (TMP) to induce human umbilical cord-derived mesenchymal stem cells (hUMSCs) to differentiate into neuron-like cells in vitro. Human umbilical cords from full-term caesarean section patients were used to obtain hUMSCs by collagenase digestion after removal of the umbilical artery and vein. The surface antigen expression profile of cultured hUMSCs was monitored by flow cytometry. After amplification, cells of the 5th passage were divided into experimental groups A-C treated with TMP at 4.67, 2.34 and 1.17 mg/ml, respectively, in low glucose-Dulbecco's Modified Eagle's Medium (L-DMEM) (induction medium), while group D (control) was exposed to L-DMEM culture medium only. Differentiation of hUMSCs into neuron-like cells and morphological changes were observed every 0.5 h with an inverted phase contrast microscope for 6 h. After the 6-h induction period, proportions of cells expressing neuronal markers neuron-specific enolase (NSE), neurofilament protein (NF-H) and glial fibrillary acidic protein (GFAP) were detected by immunohistochemistry. The optimal concentration of TMP was selected on the basis of neuron-like cell positive rate. Western blotting and RT-polymerase chain reaction were applied to detect the expression of NSE, NF-H, and GFAP of the group of optimal concentration in each point-in-time. Results showed that most primary cells were adherent 12 h after seeding and first appeared as diamond or polygon shapes. Thereafter, they gradually grew into long spindle-shaped cells and finally in a radiating or swirling pattern. The cells maintained a strong proliferative capacity after continuous passage. Flow cytometry analysis of cultured hUMSCs at the 3rd, 5th and 10th passages expressed CD73, CD90 and CD105, but not

CD11b, CD19, CD34, CD45 or human leukocyte antigen-DR. After 6 h of TMP treatment, typical neuron-like cells with many protrusions connected into a net-like pattern were observed in all experimental groups. These neuron-like cells were positive for NSE and NF-H, but negative for GFAP. Among the tested treatment groups, group A with TMP at 4.67 mg/ml had the highest expression of NSE and NF-H. By contrast, no change was found after induction in the control group. The mRNA expression of cells expressing neuronal markers as well as GAPDH was observed, with the relative NSE transcript levels of 0, 1.303 ± 0.031 , 1.558 ± 0.025 , 1.927 ± 0.019 and 2.415 ± 0.033 after 0, 1, 2, 4 and 6 h of treatment, respectively; the mRNA expression of NF-H was 0, 1.429 ± 0.025 , 1.551 ± 0.024 , 1.930 ± 0.042 and 1.398 ± 0.014 after 0, 1, 2, 4 and 6 h of treatment, respectively. There was no expression of GFAP before or after induction and all the groups showed high expression of GAPDH at each time point. Protein expression was also observed on cells expressing neuronal markers as well as GAPDH at each time point. The protein expression of NSE was 0, 0.717 ± 0.097 , 0.919 ± 0.056 , 1.097 ± 0.143 and 1.157 ± 0.055 in proper order; the protein expression of NF-H was 0, 0.780 ± 0.103 , 0.973 ± 0.150 , 1.053 ± 0.107 and 0.753 ± 0.094 in proper order. There was no expression of GFAP before or after induction, and all the groups showed high expression of GAPDH at each tested time point. Our results demonstrated that TMP can induce hUMSCs to differentiate into neuron-like cells effectively with the optimal concentration of 4.67 mg/ml. After induction, the NSE and NF-H of the neuron-like cells were positive, but the GFAP-2 was negative." As taken from Nan C et al. 2017. *Int. J. Oncol.* 48(6), 2287-94. PubMed, 2018

"Tetramethylpyrazine (TMP) has anti-inflammatory effects and is used to treat cerebral ischemic injury, but the mechanism of TMP on neural protection is not clear. Trigeminal neuralgia (TN) is a facial pain syndrome that is characterized by paroxysmal, shock-like pain attacks located in the somatosensory distribution of the trigeminal nerve. P2X3 receptor plays a crucial role in facilitating pain transmission. The present study investigates the effects of TMP on trigeminal neuralgia transmission mediated by P2X3 receptor of the trigeminal ganglia (TG). Chronic constriction injury of the infraorbital branch of the trigeminal nerve (CCI-ION) was used as a trigeminal neuralgia model. On day 15 after surgery, there was a significant decline in the mechanical hyperalgesia threshold in the territory of the ligated infraorbital nerve in the TN group, and an increase in expression of P2X3 receptor in the TG of the TN group compared with the Sham group. After treatment with TMP or A-317491, the mechanical hyperalgesia threshold of TN rats was significantly higher, and expression of P2X3 receptor in the TG noticeably declined compared with the TN group. Phosphorylation of p38 and ERK1/2 in the TN group was stronger than in the Sham group. However, the phosphorylation of p38 and ERK1/2 in the TN+TMP group and TN+A-317491 group was much lower than in the TN group. TMP significantly inhibited the ATP activated currents in HEK293 cells transfected with a P2X3 plasmid. Thus, TMP might have inhibitory effects on trigeminal neuralgia by suppressing the expression of P2X3 receptor in the TG and the phosphorylation of p38 and ERK1/2 in the TG." As taken from Xiong W et al. 2017. *Brain Res. Bull.* 134, 72-78. PubMed, 2018

"BACKGROUND: Activated astrocytes release matrix metalloproteinase-2/9 (MMP-2/9) to induce central sensitization and maintain neuropathic pain. However, the mechanisms involved in the activation of MMP-2/9 on astrocytes during pain remain poorly understood. Meanwhile, there is a lack of effective treatment to inhibit the activation of MMP-2/9 on astrocytes. In this study, we aim to investigate the effect of tetramethylpyrazine (TMP), a natural compound with analgesic effects but unknown mechanisms, on MMP-2/9 in neuropathic pain. METHODS: The nociception was assessed by measuring the incidence of foot withdrawal in response to mechanical indentation in rats (n = 6). Cell signaling was assayed using western blotting (n = 6) and immunohistochemistry (n = 5). The astrocyte cell line C8-D1A was cultured to investigate the in vitro effects. RESULTS: TMP significantly attenuated the maintenance of chronic constrictive injury (CCI)-induced neuropathic pain, inhibited the activation of astrocytes, and decreased the expression of MMP-2/9. Furthermore, our results indicated that TMP could selectively suppress JNK activity but had no notable effects on ERK and p38. Our study also revealed that the effect of TMP may be dependent on the inhibition of TAK1. CONCLUSIONS: Inhibition of astrocyte activation in the spinal cord by

tetramethylpyrazine may have utility in the treatment of CCI-induced neuroinflammation, and our results further implicate JNK-MMP-2/9 as a novel target for the attenuation of neuropathic pain.” As taken from Jiang L et al. 2017. *J. Neuroinflammation* 14(1), 174. PubMed, 2018

“Traumatic brain injury (TBI) is a diverse group of intracranial injuries resulting from external mechanical insults to the brain. While basic and clinical research for TBI has been conducted for decades, it has not identified cost-effective medical interventions for treating TBI. Tetramethylpyrazine (TMP), which is derived from the Chinese herb, *Ligusticum chuanxiong* Hort (Chuan Xiong), has been clinically used for treating ischemic brain injury for years. However, whether TMP could provide effective benefits for improving the outcomes following TBI is unknown. In the present study, using controlled cortical impact (CCI) injury to create an animal model of TBI, the potential effects of TMP on improving blood-brain barrier (BBB) permeability in the early phase of the secondary injury, as well as the splenic anti-inflammatory activities, were evaluated. Cognitive functions were also assessed by Morris water maze trials following TBI. Results demonstrated that, at 24 h after CCI injury, BBB permeability was significantly reduced ($P<0.05$) by the application of TMP. In addition, within 24 h after CCI injury, the plasma levels of interleukin (IL)- 1β and tumor necrosis factor (TNF)- α , and protein and mRNA expression levels of IL- 1β and TNF- α in the spleen were significantly lowered by TMP ($P<0.05$). Furthermore, within 24 h after CCI injury, the activation of the splenic anti-inflammatory effects mediated by nicotinic acetylcholine receptor $\alpha 7$ (nAChR $\alpha 7$) stimulation were significantly enhanced by TMP ($P<0.05$). Additionally, impaired spatial memory acquisition and consolidation were significantly improved by TMP after CCI injury ($P<0.05$). Together, in light of these data, in the treatment of TBI, TMP could effectively reduce BBB permeability, which may be closely associated with the enhanced splenic anti-inflammatory effects activated by nAChR $\alpha 7$ stimulation, and potentially improve cognitive recovery concerning spatial learning and memory.” As taken from Wang A et al. 2017. *Exp. Ther. Med.* 14(3), 2392-2400. PubMed, 2018

“OBJECTIVE: To evaluate the regulation effect of tetramethylpyrazine on microRNA-214-3p (miR-214-3p) in the spinal cord injury (SCI) rats model and to elucidate the neuroprotective effect and its mechanism of tetramethylpyrazine after SCI. METHODS: Ten Sprague-Dawley rats were used to establish the SCI rats model, and the expression levels of miR-214-3p and Bcl2l2 were detected by qRT-PCR and Western blotting at 7 days post-SCI. BBB scoring test was performed to evaluate the motor functional recovery at 21 days post-SCI. Twenty-five SCI rats were randomly assigned to five groups: SCI negative control (NC) group, tetramethylpyrazine (TMP) group, miR-214-3p agomir group, TMP/agomir group and the sham group. The rats were given a two-week injection treatment with or without TMP. The expression levels of miR-214-3p, Bcl2l2, Bax and caspase 3 were measured by qRT-PCR and Western blotting at 7 days after injection. Terminal deoxynucleotidyl transferase (TdT) -mediated dUTP Nick-End Labeling (TUNEL) assay was performed to detect cell apoptosis in vivo. Luciferase activity was measured to verify the miR-214-3p target site in the 3'-UTR of Bcl2l2 mRNA. TMP treatment was also performed to injure primary cultured neuron cells and cell apoptosis in vitro was determined by flow cytometry. RESULTS: MiR-214-3p was up-regulated while anti-apoptotic protein Bcl2l2 was downregulated post-SCI. TMP inhibited the apoptosis in vivo via decreasing the levels of miR-214-3p and increasing the expression level of Bcl2l2. A potential target site of miR-214-3p in the 3'UTR of Bcl2l2 mRNA was identified and validated by luciferase reporter assay. Furthermore, TMP could effectively inhibit neuron cells apoptosis in vitro. CONCLUSIONS: TMP alleviated neural apoptosis in injured spinal cord via down-regulation of miR-214-3p.” As taken from Fan Y and Wu Y. 2017. *Biomed. Pharmacother.* 94, 827-833. PubMed, 2018

“BACKGROUND: Traumatic brain injury (TBI) is a public health issue. As the major complaint in 51% of TBI patients, chronic pain is an important aspect in TBI treatment. Tetramethylpyrazine (TMP) is an important compound in Ligustrazine, an analgesic drug in traditional Chinese medicine, but its potential in relieving pain symptom in TBI has not been tested. We established a TBI mouse model with controlled cortical impact (CCI), and measured periorbital hypersensitivity with von Frey monofilaments. We examined activated microglia and astrocytes and the levels of substance P

(SP) and inducible isoform of nitric oxide synthase (iNOS) with immunohistochemistry, measured mRNA and protein levels of proinflammatory cytokines with qPCR and enzyme-linked immunosorbent assay, respectively. Western blot was employed to detect molecules in NF- κ B signaling pathway. RESULTS: TMP significantly attenuated periorbital hypersensitivity in TBI mice. Within 3 days after CCI, TMP attenuated activation of microglia and astrocytes, levels of SP, iNOS, and CGRP in trigeminal pathway, and levels of proinflammatory cytokines (including IL-6, TNF- α , IL-12). In isolated microglia, TMP attenuated the effects of lipopolysaccharide on the phosphorylation of cytoplasmic IKK α / β and IKB- α , and levels of nucleic p65. CONCLUSION: TMP reversed periorbital hypersensitivity by limiting neuroinflammation at the primary stage of TBI, and could be a promising drug for pain treatment in TBI." As taken from Wang Z et al. 2017. J. Inflamm. (Lond.) 14, 13. PubMed, 2018

"BACKGROUND: Transplantation of bone marrow-derived mesenchymal stem cells (BMSCs) is one of the new therapeutic strategies for treating ischemic stroke. However, the relatively poor migratory capacity of BMSCs toward infarcted regions limited the therapeutic potential of this approach. Pharmacological preconditioning can increase the expression of CXC chemokine receptor 4 (CXCR4) in BMSCs and enhance cell migration toward the injury site. In the present study, we investigated whether tetramethylpyrazine (TMP) preconditioning could enhance BMSCs migration to the ischemic brain and improve functional recovery through upregulating CXCR4 expression. METHODS: BMSCs were identified by flow cytometry analysis. BMSCs migration was evaluated in vitro by transwell migration assay, and CXCR4 expression was measured by quantitative reverse transcription-polymerase chain reaction and western blot analysis. In rats with focal cerebral ischemia, the neurological function was evaluated by the modified neurological severity score, the adhesive removal test and the corner test. The homing BMSCs and angiogenesis were detected by immunofluorescence, and expression of stromal cell-derived factor-1 (SDF-1) and CXCR4 was measured by western blot analysis. RESULTS: Flow cytometry analysis demonstrated that BMSCs expressed CD29 and CD90, but not CD34 and CD45. TMP pretreatment dose-dependently induced BMSCs migration and CXCR4 expression in vitro, which was significantly inhibited by AMD3100, a CXCR4 antagonist. In rat stroke models, we found more TMP-preconditioned BMSCs homing toward the infarcted regions than nonpreconditioned cells, leading to improved neurological performance and enhanced angiogenesis. Moreover, TMP-preconditioned BMSCs significantly upregulated the protein expression of SDF-1 and CXCR4 in the ischemic boundary regions. These beneficial effects of TMP preconditioning were blocked by AMD3100. CONCLUSION: TMP preconditioning enhances the migration and homing ability of BMSCs, increases CXCR4 expression, promotes angiogenesis, and improves neurological performance. Therefore, TMP preconditioning may be an effective strategy to improve the therapeutic potency of BMSCs for ischemic stroke due to enhanced BMSCs migration to ischemic regions." As taken from Li L et al. 2017. Stem Cell Res. Ther. 8(1), 112. PubMed, 2018

"Trigeminal neuralgia (TN) is a kind of recurrent transient and severe pain that is limited to the trigeminal nerve in one or more branches. The clinical incidence of TN is high, which seriously affects the quality of life of the patients and is difficult to cure. The present study investigated the effects of tetramethylpyrazine (TMP) on TN induced by chronic constriction injury of the infraorbital nerve (ION-CCI) in rats. Adult male Sprague-Dawley rats were randomly assigned to four groups: sham, sham treated with TMP (Sham+TMP), TN model (TN), and TN treated with TMP (TN+TMP). The rat TN model was established by ION-CCI and TMP (50 mg/kg) was injected intraperitoneally once a day for 2 weeks after operation. The mechanical response threshold was tested by the electronic von Frey filaments. The expression of CGRP in the trigeminal ganglia (TGs) of rats on the operative side was detected by RT-PCR, immunohistochemical staining and Western blot. In 15 days after operation, TN group showed a robust decrease in mechanical response threshold as compared with sham group. From day 9 to day 15 after operation, TMP treatment significantly suppressed the TN-induced mechanical hyperalgesia ($P < 0.05$). On day 15 after operation, RT-PCR, immunohistochemical staining and Western blot analysis showed an obvious increase in expression level of CGRP in TGs of TN group compared with sham group, which was

downregulated by TMP treatment ($P < 0.05$). These results suggested that TMP might have a therapeutic potential for the treatment of TN through regulating CGRP expression in the TGs." As taken from Tan MX et al. 2017. Sheng Li Xue Bao 69(1), 89-95. PubMed, 2018

"Objective To investigate the effects of tetramethylpyrazine (TMP) on experimental autoimmune myasthenia gravis (EAMG) in rats and explore the possible immune regulation mechanism. Methods Lewis rats were randomly divided into 4 groups: control group, EAMG group, TMP low-dose group (TMP-L, 10 mg/kg) and TMP high-dose group (TMP-H, 20 mg/kg). Except the control group, the other 3 groups were subjected to EAMG modeling. The body mass was determined and the symptoms of muscular weakness in rats were scored by Lennon EAMG criteria. The expressions of acetylcholine receptor (AChR), IgG and C3 in neuromuscular junctions were examined by immunofluorescence. The levels of R97-116 antibody (IgG1, IgG2a, IgG2b), tumor necrosis factor α (TNF- α), interferon γ (IFN- γ) and interleukin 10 (IL-10) were detected by ELISA. Results TMP decreased the scores in the evaluation of the symptoms of muscle weakness in EAMG rats. Moreover, TMP administration significantly inhibited AChR expression and increased IgG and C3 expression in neuromuscular junctions of EAMG rats. In addition, after TMP treatment, the levels of IgG1, IgG2a and TNF- α were remarkably reduced, while IL-10 level was elevated. However, there was no significant change in serum levels of IgG2b and IFN- γ . Conclusion TMP can exert inhibitory effects on Th1, Th2 and B cells and modulate the levels of IgG1, IgG2a, TNF- α and IL-10. All these effects contribute to relieve the symptoms of muscle weakness in EAMG rats." As taken from Ren Y et al. 2016. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 32(11), 1471-1474. PubMed, 2018

"BACKGROUND: Spinal cord injury (SCI) is a major public health issue that leads to neurological dysfunctions and morbidities in patients. Tetramethylpyrazine (TMP) plays a neuroprotective role in SCI; however, the underlying mechanism has not been fully elucidated. OBJECTIVE: In the present study, we aimed to investigate the mechanisms and therapeutic effects of TMP on SCI. METHODS: A contusion SCI model was established that used a modified Allen's method. In the TMP group, TMP (200 mg/kg) was injected daily for 5 days post-injury, while in the Negative Control (NC) group, an equal volume of normal saline was injected. Hindlimb motor function was evaluated using the Basso, Beattie, Bresnahan (BBB) scale. The effects of TMP on protein levels of the matrix metalloproteinases 2 (MMP2) and 9 (MMP9), Bax and cleaved caspase-3 were determined by western blotting. Apoptotic changes in vascular endothelial cells were evaluated using immunofluorescence and TUNEL staining. Alterations in 3D vessel morphology after treatment with TMP were assessed by synchrotron radiation micro-CT (SR μ CT). RESULTS: TMP treatment significantly improved recovery in hindlimb motor function and attenuated vascular endothelial cell apoptosis in rats with SCI. Additionally, TMP treatment markedly decreased the protein levels of MMP2 and MMP9, pro-apoptotic bax and cleaved caspase-3 while promoting angiogenesis, as evidenced by vessel visualization using SR μ CT. CONCLUSION: These results indicate that TMP attenuated SCI-induced neurological impairments by the down-regulation of the expression of MMP2 and MMP9 proteins, the inhibition of vascular endothelial cell apoptosis, and the promotion of angiogenesis." As taken from Hu JZ et al. 2017. Curr. Neurovasc. Res. 14(2), 110-116. PubMed, 2018

"Antiepileptic drugs (AEDs) are the primary agents prescribed for clinical management of limbic epilepsy. However, high incidence of pharmacoresistance and a limited armory of drugs for inhibiting the pathological progression of epilepsy pose major obstacles to managing epilepsy. Here, we investigated the effect of tetramethylpyrazine (TMP), the main bioactive alkaloid isolated from the oriental medicine Ligusticum chuanxiong Hort., against the epileptogenesis progression of acute hippocampal and corneal (6 Hz) electrical kindling models of TLE. TMP dose-dependently limited the progression of seizures and reduced the after-discharge duration (ADDs) in a hippocampal mouse kindling model. Mice treated with TMP (20, 50 mg/kg, i.p.) remained in stage 1 of epileptic progression for a protracted period, requiring additional stimulation to induce stages 2-5 epileptic phenotypes. TMP (50 mg/kg) also inhibited 6 Hz corneal kindling progression. In contrast, TMP did not reverse the phenotypes induced in a generalized seizures (GS) model, or the maximal

electroshock (MES) or pentylenetetrazole (PTZ)-induced models of epilepsy. Furthermore, patch clamp recordings revealed no effect of TMP (10 μ M) on CA1 hippocampal neurons' intrinsic properties but suppressed the (i) frequency of spontaneous excitatory post synaptic currents (sEPSCs), (ii) paired pulse ratio (PPR), and (iii) long-term potentiation (LTP) induction in the Schaffer collateral-CA1 pathway. TMP suppressed the activity of calcium, but not sodium, channels. Taken together, these results suggest that TMP has an antiepileptogenic effect, likely through suppression of excitatory synaptic transmission by its effects on inhibition of calcium channels; these traits distinguish TMP from currently available AEDs. As mice administered TMP did not show any neurologic impairment in the object recognition and open field tests, the data support further development of TMP as a promising treatment for epilepsy." As taken from Jin Y et al. 2019b. ACS Chem. Neurosci. 10(12), 4854–4863. PubMed, 2020

Background: Ligustrazine is a traditional Chinese herbal medicine that has long been used to treat cerebral ischemic disorders. However, the molecular mechanisms of ligustrazine in cerebral ischemia/reperfusion (I/R) damage have not been clear elucidated. The aim of this study was to examine the neuroprotective mechanisms of ligustrazine in cerebral I/R.

Methods: 9 C57BL/6 mice were randomly divided to three groups: Sham group (n = 3), Middle cerebral artery occlusion (MCAO) group (n = 3), and MCAO + Ligustrazine group (n = 3). The neurological deficit score was evaluated, the cerebral infarct volume was measured by triphenylterazolium chloride (TTC) staining. Differentially expressed (DE) messenger RNAs (mRNAs), long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs) were analyzed using the R package DESeq2 based on P-value < 0.05 and Log2 [fold change (FC)] $\geq$ 2 in sham group vs MCAO group and MCAO group vs ligustrazine group by high-throughput sequencing. Function enrichment analysis, the protein-protein interaction (PPI) of neurogenesis related genes were performed. The neurogenesis related competitive endogenous RNA (ceRNA) network was constructed.

Results: The expression of circ_0008146 was considerably higher in the MCAO group than the Sham group, and ligustrazine treatment markedly decreased the expression of circ_0008146 in MCAO. Next, the circ_0008146 ceRNA network was established, including circ_0008146-miR-709-Cx3cr1 ceRNA network. Besides, real time quantitative polymerase chain reaction (RT-qPCR) assay identified that miR-709 expression was considerably lower and Cx3cr1 expression was higher in the MCAO group than Sham group, and ligustrazine treatment markedly increased the miR-709 expression and reduced Cx3cr1 expression in MCAO. Further, silencing of circ_0008146 inhibited the concentration of Interleukin 6 (IL-6), Tumor Necrosis Factor alpha (TNF- α) and reduced neuron cell death and up-regulated miR-709 expression and down-regulated Cx3cr1 expression in Lipopolysaccharide (LPS) induced BV-2 cells. Dual-Luciferase reporter gene assay verified that circ_0008146 targeted miR-709.

Conclusion: Ligustrazine targets circ_0008146/miR-709/Cx3cr1 axis to inhibit cell apoptosis and inflammation after cerebral ischemia/reperfusion injury.

Li, L., et al. (2022). Ligustrazine exerts neuroprotective effects via circ_0008146/miR-709/Cx3cr1 axis to inhibit cell apoptosis and inflammation after cerebral ischemia/reperfusion injury.

Sevoflurane, a commonly used anesthetic, has been found to cause neural stem cell (NSC) injury, thereby contributing to neurocognitive impairment following general anesthesia. Tetramethylpyrazine (TMP), one of the most widely used medicinal compounds isolated from a traditional Chinese herb, possess neuroprotective activity. However, its effect on sevoflurane-induced NSC injury remains unclear. NSCs were pretreated with indicated concentrations of TMP for 2 h and then exposed to sevoflurane for 6 h. Cell injury was measured using lactate dehydrogenase (LDH) release assay. Cell viability and proliferation were detected by cell counting kit-8 (CCK-8) assay and 5-bromo-2'-deoxyuridine (BrdU) labelling, respectively. Apoptotic cells were detected using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. The levels of cleaved caspase-3, phosphorylated protein kinase B (Akt) and phosphorylated glycogen synthase kinase-3 β (GSK-3 β) were detected by western blotting. Our results showed exposure to sevoflurane decreased the viability and proliferation of NSCs, while TMP preserved NSC viability

and proliferation after sevoflurane exposure. In addition, the expression of cleaved caspase-3 and TUNEL positive cells were markedly decreased in TMP-treated NSCs compared with the control. Furthermore, pretreatment with TMP significantly increased the levels of phosphorylated Akt and GSK-3 β in sevoflurane-injured NSCs. However, an upstream inhibitor of Akt, LY294002 abolished the protective of TMP on the cell viability of NSCs. In conclusion, these findings indicate that TMP protects NSCs from sevoflurane-induced toxicity through Akt/GSK-3 β pathway.

Feng, Y., et al. (2022). Tetramethylpyrazine protects neural stem cells against sevoflurane-induced toxicity through Akt/GSK-3 β pathway. *Metabolic brain disease*, 37(7), 2457–2466.

Angiogenesis contributes to plaque instability in atherosclerosis and further increases cardio-cerebrovascular risk. Circular RNAs (circRNAs) are promising biomarkers and potential therapeutic targets for atherosclerosis. Previous studies have demonstrated that tetramethylpyrazine (TMP) and paeoniflorin (PF) combination treatment (TMP-PF) inhibited oxidized low-density lipoprotein (ox-LDL)-induced angiogenesis in vitro. However, whether circRNAs regulate angiogenesis in atherosclerosis and whether TMP-PF can regulate angiogenesis-related target circRNAs in atherosclerosis are unknown. In this study, human RNA sequencing (RNA-seq) data were analysed to identify differentially expressed (DE) circRNAs in atherosclerosis and to obtain angiogenesis-associated circRNA-microRNA (miRNA)-messenger RNA (mRNA) networks. Target circRNA-related mechanisms in angiogenesis in atherosclerosis and the regulatory effects of TMP-PF on target circRNA signalling were studied in ox-LDL-induced human umbilical vein endothelial cells (HUVECs) by cell proliferation, migration, tube formation, and luciferase reporter assays, real-time quantitative polymerase chain reaction (RT-qPCR) and Western blotting. A novel circRNA (circular stimulator of chondrogenesis 1, circSCRG1) was initially identified associated with angiogenesis in atherosclerosis, and circSCRG1 silencing up-regulated miR-1268b expression, increased nuclear receptor subfamily 4 group A member 1 (NR4A1) expression and then promoted ox-LDL-induced angiogenesis. TMP-PF (1 μ mol/L TMP combined with 10 μ mol/L PF) up-regulated circSCRG1 expression, mediated miR-1268b to suppress NR4A1 expression and then inhibited ox-LDL-induced angiogenesis. However, circSCRG1 silencing abolished the inhibitory effects of TMP-PF on ox-LDL-induced angiogenesis, which were rescued by the miR-1268b inhibitor. In conclusion, circSCRG1 might serve as a new target regulating angiogenesis in atherosclerosis via the circSCRG1/miR-1268b/NR4A1 axis and TMP-PF could regulate the circSCRG1/miR-1268b/NR4A1 axis to inhibit angiogenesis in atherosclerosis in vitro, indicating a novel angiogenesis signalling circSCRG1/miR-1268b/NR4A1 pathway in atherosclerosis and the regulatory effects of TMP-PF, which might provide a new pharmaceutical strategy to combat atherosclerotic plaque instability.

Yuan, R., et al. (2023). Identification of a Novel Angiogenesis Signalling circSCRG1/miR-1268b/NR4A1 Pathway in Atherosclerosis and the Regulatory Effects of TMP-PF In Vitro.

(...)The impact of ligustrazine on pain hypersensitivity was tested, and the results revealed that ligustrazine, but not DMSO, alleviated allodynia in CM rats. Next, to verify the possibility of ligustrazine and D2R integration, molecular docking of ligustrazine on the D2R protein was performed using AutoDock 4.2. The results suggest that D2R has a binding site with ligustrazine, and the best binding energy value was -4.24 kcal/mol. Interestingly, ligustrazine markedly blocked ROS overproduction in vitro. However, under normal conditions, neither ligustrazine nor quinpirole treatment affected neuronal ROS production or the mitochondrial membrane potential. Furthermore, additional data showed that ligustrazine and quinpirole did not show significant differences in their ROS scavenging ability. The above results confirmed the palliative effect of ligustrazine on CM-related pain sensitization, suggesting that medicines targeting D2R may have great potential in CM treatment.(...) In recent studies, ligustrazine has shown great potential in the treatment of neuroinflammation-related disorders, and it can significantly relieve some types of neuropathic pain. In this paper, we confirmed that ligustrazine effectively relieved migraine-like pain behaviors. Most importantly, ligustrazine also inhibited ROS overproduction and alleviated decreased mitochondrial potential, emphasizing the important role of TCM antioxidative therapies in the management of migraine headaches. Although ligustrazine has shown great potential in

relieving migraine and neuroinflammation, the clinical treatment of migraine is mostly based on the use of mixed formulations of several Chinese herbs, mainly Chuanxiong, Astragalus, Tianma, etc., and there have been reports suggesting synergistic or facilitating effects between ligustrazine and other Chinese herbal ingredients. As taken from Zhang W et. Al. 2024. Antioxidants (Basel).

Objective: To evaluate the effects of Ligustrazine (Lig) on nitroglycerin-induced migraine and explore the mechanism through the mitochondria-inflammation pathway. **Methods:** Rats were divided into control, model, Lig(50 mg/kg) + Erastin, Lig(100 mg/kg), Lig(50 mg/kg), and Zolmitriptan groups. Nitroglycerin (NTG) was administered through injection to trigger a migraine. The following parameters were measured: mechanical pain threshold, mitochondrial morphology, levels of reactive oxygen species (ROS), mitochondrial membrane potential (MMP), Adenosine triphosphate (ATP), and Nitric oxide (NO). The neuronal nitric oxide synthase (nNOS), transient receptor potential A1 (TRPA1), interleukin 1 beta (IL-1 β), nuclear factor-kappaB (NF- κ B), and calcitonin gene-related peptide (CGRP) were detected by Western blotting and immunohistochemistry. **Results:** Compared with the model group, the Lig(100 mg/kg) and Lig(50 mg/kg) groups increased mechanical pain threshold as well as improved abnormal mitochondrial morphology. Moreover, compared with the model group, the Lig(100 mg/kg) and Lig(50 mg/kg) groups demonstrated reduced levels of ROS, and NO, and increased MMP, and ATP. Lig(100 mg/kg) and Lig(50 mg/kg) groups reduced inflammation and oxidative stress by inhibiting certain gene expressions. When Erastin was injected, the effectiveness of Lig decreased, indicating that Lig's therapeutic effect was related to the extent of mPTP opening. **Conclusion:** The mitochondria-inflammation pathway plays a critical role in regulating migraine. Lig exerts anti-migraine effects primarily by modulating the mitochondria-inflammation pathway providing a novel perspective on migraine research that is beneficial for its clinical application. As taken from Wang Y et. Al. 2025. Neurosci Lett.

Hypothesis/purpose: This study aims to investigate the pharmacological effect and mechanism of LGZ in migraine. **Study design/methods:** A mouse model of migraine was induced by nitroglycerin (NTG), and LPS/IFN- γ stimulated microglial cell model was conducted to investigate neuroinflammation, the paracrine interactions between microglia and neurons were determined by the co-culture system, and the effect of LGZ on stability of SIRT1 protein was measured by cellular thermal shift assay (CETSA). Whilst, the SIRT1 inhibitor EX527 was used alone or co-treatment with LGZ in vitro or in vivo. **Results:** LGZ significantly attenuated migraine-like behaviors in NTG-induced mice, and ameliorated neuroinflammation and related oxidative damage in brain tissue, but co-treatment with SIRT1 inhibitor EX527 abolished the protective effects of LGZ. Mechanistically, LGZ mitigated neuroinflammation by upregulating SIRT1 expression and subsequently inhibiting the activation of NF- κ B pathway in microglia. CETSA indicated that LGZ significantly maintained the stability of SIRT1 protein in microglia. While, in the co-culture system, culture medium from LPS/IFN- γ -treated microglia exacerbated neuronal damage and oxidative stress, which was suppressed by treating LPS/IFN- γ -induced microglia with LGZ, this effect might be related to the activation of Nrf2 signals in neurons. Notably, SIRT1 inhibitor EX527 abrogated the effects of LGZ both in vitro and in vivo. **Conclusion:** Consequently, SIRT1 might be an important pharmacological target of LGZ, which attenuates migraine associated neuroinflammation and oxidative stress by interfering the crosstalk between microglia and neurons, thereby relieving migraine. As taken from Chen Y et. Al. 2024. Phytomedicine.

Objective: The inflammatory response is acknowledged as a crucial pathological aspect of spinal cord injury (SCI). Tetramethylpyrazine (TMP) has been demonstrated to possess neuroprotective properties within the central nervous system via its anti-inflammatory mechanisms. This study aims to investigate the molecular mechanism by which TMP alleviates SCI from an anti-inflammatory standpoint. **Methods:** The SCI model was established using the MASCIS impactor device. The Basso-Beattie-Bresnahan (BBB) locomotor rating scale was utilised to assess rat locomotion. Nissl and Golgi staining were used to observe neuron and dendritic spine morphology, respectively. A transmission electron microscope was used to observe the microcosmic morphology of the axon. ELISA kits were used to measure the concentrations of IL-1 β and IL-18 in the spinal cord.

Immunofluorescence staining was used to detect P2X7R+/IBA-1+ cells, and Western blot and RT-qPCR were used to analyze the protein and mRNA expression of P2X7R in the spinal cord. Additionally, Western blot was used to detect NLRP3 and Cleaved-Caspase-1 (p20), the critical proteins in the NLRP3 inflammasome pathway. Results: TMP ameliorated the microcosmic morphology of the axon and had an inhibitory effect on the concentrations of IL-1 β and IL-18 after SCI. Furthermore, TMP inhibited the expression of both P2X7R and critical proteins of the NLRP3 inflammasome pathway on microglia after SCI. The aforementioned effects of TMP exhibit similarities to those of BBG (P2X7R antagonist); however, they can be effectively reversed by BzATP (P2X7R activator). Conclusion: TMP alleviated SCI via reducing tissue damage, neuroinflammation, and the expression of P2X7R, NLRP3, IL-1 β , and IL-18. As taken from Fan X et. Al. 2024. Eur J Pharmacol.

In a separate study, researchers found that TTMP treatments effectively relieved the symptoms of Alzheimer's disease through changing the expression of relevant proteins (related to oxidative phosphorylation) in the hippocampus of mice. Similarly, due to the ability to increase the level of synapse-related proteins in the hippocampus and relieve dendritic and spine deficits, TTMP is capable of alleviating anxiety, improving cognitive ability, and reducing memory impairments. The antioxidant, anti-inflammatory, and neuro-protective activity of TTMP can contribute to treat spinal cord injury. Lin et al. established a middle cerebral artery occlusion rat model to study the mechanism of TTMP in treating ischemic stroke. The results suggested that TTMP can improve neurological function through synaptic ultra-structure remodeling and by increasing synaptophysin levels. Similarly, subsequent research found that TTMP can ameliorate neurological functional recovery and enhance endogenous neurogenesis and angiogenesis of rats with subacute ischemia. As taken from Liu X et. Al. 2024. Molecules.

Objective: To investigate the effect and mechanism of Tetramethylpyrazine (TMP) in treating Spinal Cord Injury (SCI) using network pharmacology analysis and animal experiments. Methods: This study was based on public databases, including PharmMapper, BATMAN-TCM, and STRING, as well as KEGG pathway analysis and other methods of network pharmacology were used to preliminarily explore the molecular mechanism of TMP in the treatment of SCI. Using a mouse SCI compression injury model, the efficacy of TMP was evaluated, and the expression of predictive targets on the PI3K/AKT and MAPK signaling pathways was measured using Western blotting and q-PCR. Results: Network pharmacology analysis showed that TMP may exert therapeutic effects through the MAPK and PI3K/AKT signaling pathways. In animal experimental validation studies, it was shown that after treatment with TMP, the hind limb motor function scores and ramp test scores of the TMP-treated mice improved significantly. HE staining showed that after treatment with TMP, cavities decreased, fewer glial cells proliferated, and fewer inflammatory cells infiltrated; Nielsen staining showed less neuronal loss. Western blot studies showed that compared with the model group, expression of RAS, ERK1/2, RAF1, PI3K, and p-AKT proteins in the spinal cord tissue of mice treated with high-dose TMP was significantly lower. Accordingly, q-PCR studies showed that compared with the model group, the expression levels of RAS, ERK1/2, RAF1, PI3K, and p-AKT genes in the spinal cords of mice in the high-dose TMP group were significantly lower. Conclusion: TMP exhibits a good neuroprotective effect after SCI, which may be related to inhibition of the MAPK and PI3K/AKT signaling pathways. As taken from Qi G et. Al. 2024. J Chem Neuroanat.

Ligustrazine, a component of total alkaloids, activates the PI3K/Akt pathway, thereby enhancing the eNOS/NO signaling pathway to increase NO production and protect neurological function (Ding et al., 2019). This component also alleviates neurological damage by regulating the NR2B-ERK/CREB pathway, inhibiting neuronal damage, and reducing Ca²⁺ influx (Tang et al., 2023). As taken from Chen J et. Al. 2025. J Ethnopharmacol.

Herein, we investigated whether tetramethylpyrazine pretreatment could alleviate the impairment of learning and memory and its underlying mechanism in sevoflurane-exposed neonatal rats. Postnatal 7-day Sprague-Dawley (SD) rats or primary hippocampal neurons were pretreated with tetramethylpyrazine and then exposed to sevoflurane. The terminal deoxynucleotidyl transferase-

mediated dUTP nick end labeling (TUNEL) and lactate dehydrogenase (LDH) assays were used to detect neuronal injury. Learning and memory function were evaluated by novel object recognition and Morris water maze tests. Long-term potentiation (LTP) was recorded to evaluate synaptic plasticity electrophysiologically in the hippocampal slices. Golgi-Cox staining or PSD95 immunochemistry was used to detect the morphology of dendritic spines. Western blotting was employed to assess the expressions of cleaved Caspase-3, PSD95, N-methyl-D-aspartate receptor (NMDAR) subunits NMDAR1, NMDAR2A and NMDAR2B in the hippocampus or cultured neurons. It was found that neonatal exposure of sevoflurane impaired learning and memory, increased neuronal apoptosis, altered the morphology of dendritic spines, upregulated the expressions of NMDAR2A and PSD95, and induced LTP deficits. Pretreatment with tetramethylpyrazine not only alleviated impairment of learning and memory, but also improved sevoflurane-induced changes in neuronal damage, dendritic spine morphology, NMDAR2A and PSD95 expressions, as well as LTP. These findings indicated that pretreatment with tetramethylpyrazine alleviated the impairment of learning and memory induced by sevoflurane through improvement of hippocampal synaptic plasticity in neonatal rats. As taken from Wang K et. Al. 2025. Neuroscience.

Previous studies have demonstrated that tetramethylpyrazine (TMP) can enhance the recovery of motor function in spinal cord injury (SCI) rats. (...) We conducted RNA sequencing with a network pharmacology strategy to predict the targets and mechanism of TMP for SCI. The modified Allen's weight-drop method was used to construct an SCI rat model. The results indicated that the nuclear transfer factor- κ B (NF- κ B) pathway was identified through the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, and an inflammatory response was identified through the Gene Ontology (GO) enrichment analysis. Tumor necrosis factor (TNF) was identified as a crucial target. Western blotting revealed that TMP decreased the protein expression of TNF superfamily receptor 1 (TNFR1), inhibitor κ B- α (I κ B- α), and NF- κ B p65 in spinal cord tissues. Enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC) demonstrated that TMP inhibited TNF- α , interleukin-1 β (IL-1 β), reactive oxygen species (ROS), and malondialdehyde (MDA) expression and enhanced superoxide dismutase (SOD) expression. Histopathological observation and behavior assessments showed that TMP improved morphology and motor function. In conclusion, TMP inhibits inflammatory response and oxidative stress, thereby exerting a neuroprotective effect that may be related to the regulation of the TNFR1/I κ B- α /NF- κ B p65 signaling pathway. As taken from Liu G et. al. 2024. Toxicol Appl Pharmacol.

Zhu et al. found that injection of TMP at 1–2 h after cerebral I/R in rats could remarkably reduce infarct size and nerve injury, but the effect was dramatically decreased at 4 h. After 6 h, the beneficiary role of TMP completely vanished (Zhu et al., 2009). The results suggest the time window for TMP treatment is within 4 h after reperfusion, and early application of TMP can achieve better therapeutic effects. It is well known that destruction of the blood-brain barrier (BBB) can cause I/R injury. However, the destruction of BBB at an early stage is partially reversible. Thus, early intervention to mitigate BBB disruption has great clinical significance. TMP has been reported to reduce the permeability of BBB and maintain its integrity (Gong et al., 2019; Tan, Fu, Cheng, Meng, & Gu, 2015; Yang et al.,...) Currently, most research has focused on the inhibitory effect of TMP on the early response of the body to cerebral ischemic injury. Interestingly, recent results have suggested that TMP displays the neurorestorative effects as well. Lin et al. found that TMP significantly improved neurological function on day 7 and 14 after ischemia by regulating dendrite plasticity (Lin et al., 2015). In addition, TMP can repair nerve damage by promoting the migration of neural precursor cells (NPCs) and bone marrow-derived mesenchymal stem cells (BMSCs), which accelerates neurogenesis and angiogenesis (Kong et al., 2016; Li et al., 2019). (...) Surgery on the descending thoracic, abdominal aorta or spinal cord may cause spinal cord injury. In the animal model, surgically blocking infrarenal aortic mimics a clinical aortic occlusion in human. By using the model, a study demonstrated that TMP was able to prevent neuronal degeneration and necrosis in anterior horn motor neurons (Chen et al., 2002). In addition to infrarenal aortic blockade, balloon blockade of the thoracic aorta can also produce spinal cord I/R injury. It has been reported that TMP might exert neuroprotective effect in spinal cord I/R injury through anti-inflammation because it

could improve the expression of anti-inflammatory cytokines and inhibit the expression of pro-inflammatory cytokines (Fan et al., 2011). The protective effect of TMP on I/R injury in rabbits was dose-dependent. In addition, TMP must reach a certain concentration to protect neuronal cells (Chen et al., 2002). As taken from Qi M et. al. 2024. Pharmacol Ther.

In a recent study, a subarachnoid hemorrhage (SAH) animal model was treated with TMP, and the mitochondrial-dependent Caspase-3 apoptosis pathway was inhibited. Through direct interaction with TFAM, TMP stabilizes TFAM, leading to inhibition of the Lon protease pathway, a reduction in TFAM degradation, and an increase in mtDNA copy number. These interactions led us to investigate whether TMP can mitigate neuronal damage after the occurrence of SBI. Therefore, we established an SBI rat model and intervened by intraperitoneal injection of TMP to observe whether TMP could increase TFAM content. Can the increase of TFAM content play a role in protecting mitochondrial function, thereby reduce the production of ROS and improve neurological function?(...) In this study, it was found that after SBI, treatment with TMP increased TFAM levels, promoted mitochondrial function, reduced ROS production, and increased ATP levels. As a result, Caspase-3 was inhibited and Bcl-2 levels increased, thereby reducing brain edema and nerve cell apoptosis. Thus, we concluded that TMP played a role in brain protection. As such, TFAM could be an important future target for both preventing and treating secondary brain injury after SBI. As taken from Wang C et. Al. 2024. Iran J Basic Med Sci.

Objective: Observing the effects of Tetramethylpyrazine (TMP) on the expression of Collagen IV and Laminin in neurovascular basement membrane and the apoptosis of vascular endothelial cells, and to study the mechanism of TMP in the treatment of sciatic nerve injury. **Results:** Compared with the NS group, the TMP group had a significant increase in the sciatic nerve function index ($P < 0.01$). The miss times in TMP group was significantly lower than that in NS group ($P < 0.01$). The HE staining results of the TMP group showed irregular arrangement of some neuronal axons and Schwann cells, and more edema and rupture of cells. The proliferation of glial cells and inflammatory cells was significantly increased in TMP group. The results of immunohistochemistry showed that the expression of type IV collagen and laminin in the TMP group group was distributed around the blood vessels, vascular endothelial cells, basal membrane and glial cells after SNI. The expression of type IV collagen and laminin in TMP group increased significantly ($P < 0.05$). Immunofluorescence showed that compared with NS group, the apoptosis rate of TMP group was significantly decreased ($P < 0.01$). Flow cytometry results showed that compared with the NS group, the number of CECs in the TMP group was significantly decreased ($P < 0.01$). **Conclusions:** TMP can effectively improve the sciatic nerve functional index (SFI) of Sprague Dawley (SD) rats, enhance the proliferation of sciatic nerve vascular endothelial cells, reduce apoptosis, promote the expression of Collagen IV and Laminin in sciatic nerve microvascular basal membrane components, thereby promoting angiogenesis and improving lower limb function in rats. As taken from Li Y et. Al. 2024. BMC Neurosci.

In recent years, the pharmacological effects of TMP have been widely demonstrated in the cell models. TMP exerts an anti-neurotoxic effect on glutamate damaged PC12 cells (Tong, Wang, Wang, & Lu, 2022). Ischemia and hypoxia can lead to neuronal death, causing brain damage. Evidence has shown that TMP can reduce oxygen and glucose deprivation (OGD)-induced neuronal death via a concentration dependent manner (Shao, Wang, Liu, & Wang, 2017). TMP also exerted significant anti-inflammatory effects by inhibiting the activation of macrophages, microglia, parenchymal infiltrating neutrophils, and circulating neutrophils (Hsiao et al., 2006; Kao et al., 2013). As taken from Qi M et. al. 2024. Pharmacol Ther.

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

“We have previously demonstrated that tetramethylpyrazine (TMP) could stimulate colonic and pancreatic anion secretion. The present study investigated the signaling pathways and cellular mechanisms underlying the effect of TMP using human colonic Caco-2 cells, with permeabilized apical or basolateral membranes, in conjunction with Ussing chamber technique, intracellular cAMP

and Ca^{2+} measurements as well as competitive RT-PCR for mRNA expression of cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+} -dependent Cl^- channels (CACC). Basolateral addition of TMP induced a short circuit current ($I(\text{SC})$) response, which could be mimicked by forskolin and 3-isobutyl-1-methylxanthine (IBMX). Adenylate cyclase inhibitor, MDL12330A, and intracellular Ca^{2+} chelator, BAPTA-AM, significantly inhibited the TMP-induced $I(\text{SC})$. In basolateral membrane-permeabilized cells, TMP, as well as forskolin and IBMX, induced an $I(\text{SC})$ response, which was sensitive to MDL-12330A, H89, and specific channel blocker CFTR(inh-172), but insensitive to apical application of 4,4'-dithiocyano stilbene-2, 2'-disulfonic acid (DIDS) and basolateral pretreatment with BAPTA-AM. In apical membrane-permeabilized cells, TMP, similar to forskolin and IBMX, produced a very small current increase, which was sensitive to K^+ channel blockers, BaCl_2 and tetraethylammonium (TEA), but not Chromanol 293B and charybdotoxin (ChTX), alone or combined. However, in intact Caco-2 monolayers, the TMP-induced $I(\text{SC})$ could be partially inhibited by ChTX. TMP (5 mM) could stimulate intracellular cAMP production. Intracellular Ca^{2+} was also increased by TMP (5 mM) in both Ca^{2+} -containing and Ca^{2+} -free bathing solutions. RT-PCR showed that the expression of CFTR in Caco-2 cells was 5.2 fold higher than that of Ca^{2+} -activated Cl^- channel (CACC). In conclusion, TMP stimulates Cl^- secretion by activating cAMP and $[\text{Ca}^{2+}]_i$ signaling pathways leading to subsequent activation of apical CFTR and basolateral K^+ channels." As taken from Zhu ZX et al. Eur. J. Pharmacol. 2005. 510(3), 187-95. PubMed, 2010

"In the present study we investigated the role of Ca^{2+} in tetramethylpyrazine (TMP)-induced anion secretion in the human colonic epithelial cell line, Caco-2, using the short-circuit current ($I(\text{SC})$) technique in conjunction with intracellular Ca^{2+} measurements. The results showed that TMP-induced $I(\text{SC})$ response was significantly reduced by 58.8% and 38.3% after inhibiting Ca^{2+} -ATPase of endoplasmic reticulum (ER) with thapsigargin and mobilizing ER stored Ca^{2+} release with ATP, respectively. Conversely, thapsigargin- and ATP-evoked $I(\text{SC})$ responses were also significantly reduced by pretreatment with TMP by 43.2% and 38.5%, respectively. Conversely, removal of extracellular Ca^{2+} , apical but not basolateral, or the presence of the Ca^{2+} chelator (EGTA) significantly increased TMP-induced $I(\text{SC})$ by 47.1% and 37.8%, respectively. Similar to TMP, thapsigargin-induced current increase was also enhanced by chelating extracellular Ca^{2+} or in Ca^{2+} free solution; however, removal of extracellular Ca^{2+} did not significantly affect 3-isobutyl-1-methylxanthine (IBMX)- and forskolin-induced transepithelial current. Measurement of the intracellular concentration of free Ca^{2+} ($[\text{Ca}^{2+}]_i$) with fura-2/AM showed that TMP could induce an increase in $[\text{Ca}^{2+}]_i$ but pretreatment with TMP significantly reduced thapsigargin-evoked, but not ATP-induced, $[\text{Ca}^{2+}]_i$ increase. These results suggest that the effect of TMP on colonic anion secretion is partly mediated by TMP-increased $[\text{Ca}^{2+}]_i$ by acting on a target similar to thapsigargin. The observed inhibitory effect of extracellular Ca^{2+} on Ca^{2+} -dependent anion secretion represents a novel mechanism by which Ca^{2+} -dependent regulation of epithelial electrolyte transport may be fine-tuned by extracellular Ca^{2+} in the apical domain." As taken from Zhu ZX et al. Cell. Biol. Int. 2006. 30(6), 547-52. PubMed, 2010

"The aim of this work was to study the effects of tetramethylpyrazine (TMP) on the interaction between lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesive molecule-1 (ICAM-1) by micropipette technique. A pair of cells, i.e., a human lymphocyte or a S KW-3 cell (human T cell leukemia), LFA-1 was expressed on which cellular surfaces, and a RBC coupled with ICAM-1, were the carriers for LFA-1 and ICAM-1. The adhesion probabilities of this pair of cells were mediated by specific interactions between ICAM-1 and LFA-1. Phytohemagglutinin (PHA) stimulation resulted in the significant increase in the adhesion probability compared to the resting lymphocyte. TMP treatment can inhibit such increase and even make the adhesion probability lower than the resting state. While the LFA-1 expression has not changed significantly with PHA stimulation or with TMP treatment, which indicated that TMP inhibiting effects was realized by a possible conformation change." As taken from Zhao H et al. Clin. Hemorheol. Microcirc. 2000. 23(2-4), 145-51. PubMed, 2010

"In this study, the intraperitoneal administration of 1 mg/kg thioacetamide (TAA) produced hepatotoxicity in mice. The increase in serum SGOT and SGPT produced at 24 h by this regimen was decreased in a dose-dependent manner by coadministration of tetramethylpyrazine (TMP; 10, 25 and 50 mg/kg). A rise in serum interleukin-2 was similarly prevented. Increased concentrations of malondialdehyde (MDA) generated in vitro in liver homogenates prepared from TAA-treated mice were decreased by TMP treatments. The increase in MDA produced by TAA was also prevented by in vitro addition of TMP to liver homogenates. These results suggest that part of the hepatocellular injury induced by TAA is mediated by oxidative stress caused by the action of cytokines through lipid peroxidation. TMP appears to act by preventing lipid peroxidation." As taken from So EC et al. J. Biomed. Sci. 2002. 9(5), 410-4. PubMed, 2010

"To investigate the effect of tetramethylpyrazine on hepatic/renal ischemia and reperfusion injury in rats. METHODS: Hepatic/renal function, histopathological changes, and hepatic/renal P-selectin expression were studied with biochemical measurement and immunohistochemistry in hepatic/renal ischemia and reperfusion injury in rat models. RESULTS: Hepatic/renal insufficiency and histopathological damage were much less in the tetramethylpyrazine-treated group than those in the saline-treated groups. Hepatic/renal P-selectin expression was down regulated in the tetramethylpyrazine-treated group. CONCLUSION: P-selectin might mediate neutrophil infiltration and contribute to hepatic/renal ischemia and reperfusion injury. Tetramethylpyrazine might prevent hepatic/renal damage induced by ischemia and reperfusion injury through inhibition of P-selectin." As taken from Chen JL et al. World J. Gastroenterol. 2003. 9(7), 1563-6. PubMed, 2010

"The effect of tetramethylpyrazine (TMP) on lymphocytes proliferation response (LPR) detected by 3H-TdR incorporation into murine monocytes. The results showed that TTMP at the concentration of 100-1000 micrograms/ml inhibited LPR in normal mice and B14-melanoma bearing mice in vitro the effect was dose-dependent. It was also found TTMP could stimulate LPR to Con A at the concentration of 1 micron/ml in normal mice, and at the concentration of 100-1000 micrograms/ml in B16-melanoma bearing mice. But TTMP inhibited LPR to Con A at the concentration of 100-1000 micrograms/ml in normal mice. The restoration of LPR to PHA and increasing 3H-TdR incorporation into splenocytes were found in vivo in B16-melanoma bearing mice after treatment with TTMP. The data indicates that TTMP has an immune modulation." As taken from Liu J et al. Hua Xi Yi Ke Da Xue Xue Bao. 1995. 26(2), 177-9. PubMed, 2010

"In the present study, the effect of tetramethylpyrazine (TMP) on calcium (Ca^{2+}) influx was investigated in cultured vascular smooth muscle (A7r5) cells using Fura-2 as an indicator. The increase of Ca^{2+} concentration in A7r5 cells produced by vasopressin or phenylephrine was attenuated by TMP from 0.01 micromol/L to 1 mmol/L. The decrease in the intracellular potassium concentration in A7r5 cells by TMP from 0.01 micromol/L to 10 micromol/L was characterized using PBFI/AM. Inhibitors specific to the small conductance calcium-activated potassium (SKCa) channel or the ATP-sensitive potassium (K ATP) channel abolished the actions of TMP. The obtained results indicate that the decrease of Ca^{2+} influx into A7r5 cells by TMP is mainly mediated by the opening of potassium channels." As taken from Tsai CC et al. Planta Med. 2003. 69(6), 557-8. PubMed, 2010

"To explore the effects of ligustrazine on hematopoietic reconstitution and its mechanism after bone marrow transplantation (BMT), the allogenic BMT mice were given intra-abdominal injection of 2 mg ligustrazine twice a day. On the 1st, 7th, 14th, and 28th day after BMT, peripheral blood cells and bone marrow nuclear cells (BMNC) were counted, and the histological features were evaluated. On the 7th, 14th, 21st day after BMT, CXCR4 expression on the BMNC was assayed. The results showed that peripheral blood cell counts and BMNC counts in ligustrazine-treated group on the 7th, 14th, 28th day were higher than those in BMT group ($P < 0.01$ or $P < 0.05$). The percentage of hematopoietic tissue volume, fat tissue hyperplasia and congestion and dilation degree of microvessel in ligustrazine-treated group on the 7th, 14th, 21st, 28th day was higher than those in BMT group. The CXCR4 expression levels in ligustrazine-treated group were higher than in BMT group ($P < 0.01$ or $P < 0.05$) on the 7th and 14th day, and were lower than in BMT group on

the 21st day ($P < 0.01$). It is concluded that the ligustrazine can accelerate hematopoietic reconstruction, enhance growth of hematopoietic tissues and promote the repair of microvessels. The CXCR4 expression levels on BMNC may be responsible for the effect of ligustrazine.” As taken from Fang M et al. *J. Tongji Med. Univ.* 2000. 20(2), 120-2, 125. PubMed, 2010

“BACKGROUND: In this study we investigated the effects of different dosages of ligustrazine (tetramethylpyrazine, Tmp) on soleus function and sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) activity in 14-d hindlimb-unloaded (HU) rats. METHODS: Female Sprague-Dawley rats were randomly divided into 4 groups (8 rats in each group): synchronous control (CON); HU plus intragastric water instillation (HU-W); HU plus different dosages of instilled Tmp (high: $42.53 \text{ mg} \times \text{kg}^{-1}$), HU-TmpH; low: $21.15 \text{ mg} \times \text{kg}^{-1}$), HU-TmpL). Muscle contraction force was examined in the soleus muscle. SERCA activity was assayed according to the released inorganic phosphate content. RESULTS: As expected, in HU-W, soleus peak twitch tension (Pt), peak tetanic tension (P0), time to 50% peak tension (TP50), time to peak tension (TPT), time from peak tension to 50% relaxation (RT50), and SERCA activity decreased, all compared with CON. HU-TmpH Pt and P0 values were 179% and 90% above HU-W, and 187% and 124% above HU-W in HU-TmpL, respectively. TP50 and TPT values were 148% and 80% slower than HU-W with HU-TmpH and 95% and 32% in the HU-TmpL group, respectively. RT50 was slower than HU-W by 21% in HU-TmpH; SERCA activity elevated by 56% with HU-TmpH and by 72% with HU-TmpL. CONCLUSIONS: Ligustrazine may alleviate the decrease of muscle contractile force and increase of shortening velocity in atrophied soleus, possibly by means of elevated sarcoplasmic reticulum Ca^{2+} -ATPase activity.” As taken from Li S et al. *Aviat. Space Environ. Med.* 2012. 83(11), 1049-54. PubMed, 2013

“Hepatic fibrosis represents a frequent event following chronic insult to trigger wound healing reactions with accumulation of extracellular matrix (ECM) in the liver. Activation of hepatic stellate cells (HSCs) is the pivotal event during liver fibrogenesis. Compelling evidence indicates that oxidative stress is concomitant with liver fibrosis irrespective of the underlying etiology. Natural antioxidant ligustrazine exhibits potent antifibrotic activities, but the mechanisms are poorly understood. Our studies were to investigate the ligustrazine effects on HSC activation stimulated by hydrogen peroxide (H_2O_2), an in vitro model mimicking the oxidative stress in liver fibrogenesis, and to elucidate the possible mechanisms. Our results demonstrated that H_2O_2 at $5 \mu\text{M}$ significantly stimulated HSC proliferation and expression of marker genes of HSC activation; whereas ligustrazine dose-dependently suppressed proliferation and induced apoptosis in H_2O_2 -activated HSCs, and attenuated expression of fibrotic marker genes. Mechanistic investigations revealed that ligustrazine reduced platelet-derived growth factor- β receptor (PDGF- βR) expression and blocked the phosphorylation of extracellular regulated protein kinase (ERK) and p38 kinase, two downstream effectors of PDGF- βR . Further molecular evidence suggested that ligustrazine interruption of ERK and p38 pathways was dependent on the blockade of PDGF- βR and might be involved in ligustrazine reduction of fibrotic marker gene expression under H_2O_2 stimulation. Furthermore, ligustrazine modulated some proteins critical for HSC activation and ECM homeostasis in H_2O_2 -stimulated HSCs. These data collectively indicated that ligustrazine could attenuate HSC activation caused by oxidative stress, providing novel insights into ligustrazine as a therapeutic option for hepatic fibrosis.” As taken from Zhang F et al. *Toxicol. Appl. Pharmacol.* 2012b. 265(1), 51-60. PubMed, 2013

“Hepatic stellate cell (HSC) activation is the central event during liver fibrogenesis. Metabolic syndrome characterized by hyperglycemia and hyperinsulinemia contributes to nonalcoholic steatohepatitis-associated liver fibrosis. This study was to investigate the effects of tetramethylpyrazine (TMP) on HSC activation induced by glucose and insulin (Glu/Ins) and the underlying mechanisms. Results showed that Glu/Ins significantly stimulated proliferation, invasion, adhesion, and extracellular matrix (ECM) production in HSCs. TMP inhibited HSC proliferation, invasion and adhesion, and reduced the expression of marker genes related to HSC activation in Glu/Ins-activated HSCs. Mechanistic evidence revealed that TMP reduced insulin receptor (InsR) expression and blocked the downstream phosphatidylinositol-3-kinase (PI3K)/AKT and extracellular

signal-regulated kinase (ERK) cascades, which was required for TMP attenuation of HSC activation. Moreover, TMP modulated the genes relevant to ECM homeostasis favoring ECM degradation. It could be concluded that TMP inhibited Glu/Ins-stimulated HSC activation and ECM production by inhibiting InsR-mediated PI3K/AKT and ERK pathways.” As taken from Zhang F et al. 2014. *Mol. Cell. Endocrinol.* 382(1), 197–204. PubMed, 2015

“BACKGROUND: The sepsis-induced acute liver injury majorly depends on the dysfunction of mitochondria and the loss of cellular energy. Aquaporin 8 (AQP8) can modulate water transport and osmotic swelling of mitochondria in the inner mitochondrial membrane of the liver. In this study, we explore the effects of tetramethylpyrazine (TMP) on protecting the structure of hepatocyte mitochondria and modulating the expression of AQP8. MATERIALS AND METHODS: Forty-eight rats were randomly allocated to four groups: control group receiving sham procedure, septic group receiving cecal ligation and puncture (CLP), therapeutic group receiving 60 mg/kg of ligustrazine (TMP) intravenously from caudal vein immediately after CLP, and preventive group receiving 60 mg/kg/d of ligustrazine intravenously from caudal vein for 7 d before CLP. The mitochondrial ultrastructure of rat liver was observed. The protein expression of AQP8 was assayed by Western blot. Analysis of AQP8 messenger RNA (mRNA) expression level was performed by the reverse transcription-polymerase chain reaction. The mean fluorescence intensity (MFI) of rhodamine 123 (Rh 123) was measured by flow cytometry. The serum tumor necrosis factor alpha (TNF- α) level was determined by the enzyme-linked immunosorbent assay. RESULTS: The mitochondrial ultrastructure was markedly damaged in the septic group, whereas it was lightly damaged in the therapeutic and preventive groups. Compared with the control group, the AQP8 protein expression and MFI were significantly reduced, and the steady-state AQP8 mRNA and serum TNF- α levels were increased in the septic, therapeutic, and preventive groups. Compared with the septic group, the AQP8 protein expression and MFI were increased, and the steady-state AQP8 mRNA and serum TNF- α levels were decreased significantly in the therapeutic and preventive groups. There was no significant difference in morphologic characteristics, AQP8 protein level, AQP8 mRNA level, MFI, and serum TNF- α level between the therapeutic and the preventive groups. Linear positive correlation was observed between the AQP8 protein level and the MFI of Rh 123. Linear negative correlation was observed between the AQP8 protein level or the MFI of Rh 123 and serum TNF- α level. CONCLUSIONS: TMP HAS PROTECTIVE EFFECT ON HEPATOCELLULAR MITOCHONDRIA FROM DAMAGE IN SEPSIS BY AMELIORATING THE EXPRESSION OF AQP8 PROTEIN IN LIVER MITOCHONDRIA. THE PROTECTIVE EFFECT OF TMP ON THE LIVER MITOCHONDRIA MIGHT NOT HAVE A DIFFERENCE BETWEEN USING TMP BEFORE OR AFTER THE OCCURRENCE OF SEPSIS.” AS TAKEN FROM WANG JQ ET AL. 2013. *J. SURG. RES.* 185(1), 286–93. PUBMED, 2013

“Liver fibrosis represents a frequent event following chronic insult to trigger wound healing responses in the liver. Activation of hepatic stellate cells (HSCs) is a pivotal event during liver fibrogenesis. Compelling evidence indicates that the renin-angiotensin system (RAS) takes part in the pathogenesis of liver fibrosis. Angiotensin II (Ang II), the primary effector peptide of the RAS, has been demonstrated to be a potent pro-fibrogenic molecule for HSC activation. In this study we investigated the effects of tetramethylpyrazine (TMP) on HSC activation induced by Ang II in order to elucidate the underlying mechanisms. Our results demonstrated that Ang II significantly promoted cell growth, upregulated the expression of the fibrotic markers α -smooth muscle actin (α -SMA) and α 1(I) procollagen, and enhanced the invasion capacity in HSCs. TMP inhibited proliferation and arrested the cell cycle at the G2/M checkpoint associated with altering several cell cycle regulatory proteins in Ang II-treated HSCs. TMP also modulated Bcl-2 family proteins and activated the caspase cascade leading to apoptosis in Ang II-treated HSCs. Moreover, TMP reduced the expression of α -SMA and α 1(I) procollagen at mRNA and protein levels, and these effects were associated with interference of the platelet-derived growth factor β receptor (PDGF- β R) mediated PI3K/AKT/mTOR pathway in HSCs exposed to Ang II. Furthermore, Ang II-enhanced HSC invasion capacity was diminished by TMP, which was associated with interference of PDGF-

β R/FAK signaling. These data collectively indicated that interference of PDGF- β R-mediated fibrotic pathways was involved in TMP inhibition of HSC activation caused by Ang II, providing novel mechanistic insights into TMP as a potential therapeutic remedy for hepatic fibrosis". As taken from Zhang X et al. 2014. FEBS J. 1(12), 2754-68. PubMed, 2015

"Tetramethylpyrazine (TMP) is an effective Chinese plant-derived medicine for colitis in the clinic, but the underlying molecular mechanisms of its use remain poorly understood. The purpose of this study was to investigate the mechanisms involved in its therapeutic action. METHODS: A colitis mouse model was induced by oxazolone enema. TMP was administered at 80 mg/kg/day and sulphasalazine (SASP) was used as positive control and administered at 100 mg/kg/day for the treatment of colitis. On the fourth day after enema, mice were sacrificed. The inflammatory response was assessed by the disease activity index and histology. Colon mucosa was isolated and biochemically analyzed. In addition, in vitro studies were performed to evaluate the activity of TMP in Caco-2 cells. RESULTS: Our results showed that TMP improved the colonic inflammatory status as evidenced by histological findings, as well as SASP. These effects were associated with a decrease in nucleus translocation of NF- κ B. Paired with this inhibitive activity, there was a decrease in downstream signaling, such as C-MYC, iNOS and COX-2. In vitro assays revealed that TMP inhibited NF- κ B translocation and its downstream production of inflammatory factors, such as TNF- α , IL-6 and IL-8, and that ROS production that was induced by LPS in Caco-2 cells. CONCLUSION: TMP improved the colitis induced by oxazoline, and its activity was associated with inhibition of NF- κ B translocation, and subsequent inhibition of pro-inflammatory factor production and oxidative stress". As taken from Lu Y et al. 2014. Clin. Invest. Med. 37(1), E1-9. PubMed, 2015

"In this study, we aimed at evaluating the effect of ligustrazine, a major constituent of Ligusticum wallichii from traditional Chinese medicine, on Cd-induced changes in nephrotoxicity indices. Rats were divided into four experimental groups: control; ligustrazine; Cd and ligustrazine+Cd. Cd treated alone group showed significant decreases ($P<0.05$) in body weight, renal levels of superoxide dismutase (SOD) and glutathione reductase (GR); and significant increases ($P<0.05$) in urine volume (24h), pH values, serum blood urea nitrogen (BUN), serum uric acid, kidney malondialdehyde (MDA), urinary total protein, urinary glucose, urinary lactate dehydrogenase (LDH) and urinary alkaline phosphatase (ALP). Apart from indoxyl sulfate (a uremic toxin), two newly accepted nephrotoxicity biomarkers including kidney injury molecule-1 (kim-1) and clusterin were also found to be increased. Nonetheless, all these effects induced by Cd were reversed upon treatment by ligustrazine although it failed in decreasing the concentrations of Cd in kidney and urine. Histopathological studies in Cd-treated rats exhibited renal tubule damage, which was also ameliorated by ligustrazine pretreatment. These results suggest that ligustrazine exhibits protective effects on Cd-induced nephrotoxicity. Additionally, this study also demonstrates Cd exposure induces elevated levels of indoxyl sulfate in serum and kidney, and clusterin in urine." As taken from Lan Z et al. 2014. Food Chem. Toxicol. 63, 62-8. PubMed, 2015

"Steroid-induced osteonecrosis of the femoral head (steroid-induced ONFH) is an avascular necrosis disease of bone. Tetramethylpyrazine (TMP), with significant vascular protective properties, has been widely used for the treatments of ischemic neural disorders and cardiovascular diseases. However, its role in the treatment of steroid-induced ONFH has not been evaluated. In this study, our results showed that TMP significantly decreased the ratio of empty lacuna, adipose tissue area, and adipocyte perimeter in steroid-induced ONFH rats histopathologically. TMP also reduced the levels of serum lipid dramatically by haematological examination. According to the micro-CT quantification, TMP could improve the microstructure of the trabecular bone and increases bone mineral density in steroid-induced ONFH rats. Moreover, TMP significantly increased the vessel volume, vessel surface, percentage of vessel volume, and vessel thickness of the femoral heads by micro-CT. Interestingly, the downregulation of VEGF and FLK1 proteins in the sera and necrotic femoral heads could be reversed by TMP treatment, and this was true for their mRNA expressions in femoral heads. In conclusion, these findings suggest for the first time that TMP may prevent steroid-induced ONFH and also enhance femoral head vascularization

by inhibiting the effect of steroid on VEGF/FLK1 signal pathway.” As taken from Jiang Y et al. 2015. Biomed. Res. Int. 2015, 315850. PubMed, 2016

“OBJECTIVE: Tetramethylpyrazine has been suggested to have a therapeutic effect on impaired hearing that is induced by aminoglycoside antibiotics. However, its effectiveness on streptomycin ototoxicity and its cellular mechanisms are relatively unknown. Here we investigate the protective effect of tetramethylpyrazine on streptomycin-induced ototoxicity in guinea pig cochlea. STUDY DESIGN: Prospective randomized laboratory study. SETTING: Hearing Research Laboratory of China Medical University. SUBJECTS AND METHODS: Adult guinea pigs were randomized to 4 groups. Hearing sensitivity of guinea pigs was tested by auditory brainstem response measurements before streptomycin exposure and again 10 days later. The cochlear tissues were prepared for electron microscopy and immunohistochemical staining of heat shock protein 70 (HSP70). The effect of tetramethylpyrazine on streptomycin-induced activation of caspase-3 was evaluated by Western blotting. RESULTS: Co-therapy with tetramethylpyrazine reduced a profound streptomycin-induced auditory threshold shift compared with streptomycin treatment alone ($P = .0002$ or $P = .00008$). Tetramethylpyrazine also attenuated the structural disruption in streptomycin-treated outer hair cells and marginal cells of vascular stria by transmission electronic microscopy and scanning electronic microscopy, respectively. Moreover, tetramethylpyrazine decreased the streptomycin-stimulated expressions of HSP70 and caspase-3. The correlation analysis demonstrated that HSP70 expression had a positive correlation with auditory brainstem response thresholds ($|R| = 0.6-0.9$, $P = .0073$ or $P = .0169$). CONCLUSIONS: Our data suggest that the protective effect of tetramethylpyrazine on hearing function is associated with the reduction of stress response and inhibition of apoptosis. Tetramethylpyrazine may have therapeutic potential for patients with ototoxicity diseases.” As taken from Cui C et al. 2015. Otolaryngol. Head Neck Surg. 152(5), 904-11. PubMed, 2016

“Hepatic fibrosis is concomitant with liver inflammation, which has been highlighted as significant treatment of chronic liver disease. We previously demonstrated that tetramethylpyrazine (TMP), the effective component of *Ligusticum chuanxiong* Hort, can inhibit the activation of HSCs and consequential anti-hepatic fibrosis. In this study, our work demonstrated that TMP improved liver histological architecture, decreased hepatic enzyme levels and attenuated collagen deposition in the rat fibrotic liver. In addition, TMP significantly protected the liver from CCl₄-caused injury and fibrogenesis by suppressing inflammation with reducing levels of inflammatory cytokines, including tumor necrosis factor- α (TNF- α), NLRP3, nuclear factor-kappa B (NF- κ B) and interleukin-1 β (IL-1 β). Experiments in vitro showed that TMP inhibited inflammatory cytokine expression in HSCs associated with disrupting platelet-derived growth factor-b receptor (PDGF- β R)/NLRP3/caspase1 pathway. These data collectively indicate that TMP can attenuate liver inflammation in liver fibrosis and possibly by targeting HSCs via PDGF- β R/NLRP3/caspase1 pathway. It provides novel mechanistic insights into TMP as a potential therapeutic remedy for hepatic fibrosis.” As taken from Wu X et al. 2015. IUBMB Life 67(4), 312-21. PubMed, 2016

“AIMS: Inhibiting the major features of alcoholic liver disease (ALD) such as lipid accumulation and oxidative stress is a promising strategy of treating ALD. Tetramethylpyrazine (TMP) has curative effects on various diseases. However, the effects of TMP on ethanol-induced hepatocyte injury and the related mechanisms remain unclear. The aim of this study is to elucidate the effects of TMP and the potential mechanisms in vitro. MAIN METHODS: Ethanol-stimulated LO2 cells were used as an in vitro model of ALD. Several biomarkers related to cell injury, lipid accumulation, and oxidative stress were determined to evaluate the effects of TMP. Nuclear factor erythroid 2-related factor 2 (Nrf2) expression plasmids and Nrf2 small interfering RNA (siRNA) were used to establish the role of Nrf2. KEY FINDINGS: TMP increased Nrf2 expression and nuclear translocation. TMP prevented ethanol-induced hepatocyte injury, as indicated by the enhanced cell viability, reduced activities of aspartate transaminase and alanine aminotransferase in the culture medium, and inhibition of cell apoptosis. Furthermore, TMP reduced the levels of lipid droplets, triglyceride, and total cholesterol, probably by regulating genes related to lipid metabolism. Besides, TMP alleviated ethanol-induced oxidative stress by increasing superoxide dismutase activity and the glutathione level and by

reducing the levels of reactive oxygen species and malondialdehyde. In addition, overexpression of Nrf2 enhanced the effects of TMP on cell injury, lipid accumulation, and oxidative stress, whereas Nrf2 siRNA eliminated the effects of TMP. As taken from Lu C et al. 2015. Life Sci. 141, 119-127. PubMed, 2016

“ETHNOPHARMACOLOGICAL RELEVANCE: Tetramethylpyrazine (TMP) is one of the active constituents extracted from a frequently used herb, *Ligusticum wallichii* Franchat (Chuan-Xiong in Chinese), in traditional Chinese medicine. TMP can exert multiple pharmacological actions such as anti-inflammatory, anti-oxidative damage, anti-platelet and neuroprotective effects, and its applications deserve further explored. **AIM OF THE STUDY:** This study aimed to determine the new role of TMP identified by a network pharmacology approach to alleviate the methotrexate (MTX)-induced oxidative injury and characterize their mechanism of combinational actions. **MATERIALS AND METHODS:** A network pharmacology-based screening strategy is applied for target profile prediction and pharmacological characterization of herbal compounds, which is used to guide the following in vitro and in vivo experiments. The effect of herbal compounds identified by network pharmacology approaches to reduce the toxicity of MTX was assessed by MTX-induced rat toxicity model. The potential targets of TMP in this study were evaluated using standard protocols provided by Cerep, Inc. **RESULTS:** This strategy identified TMP from *Ligusticum wallichii* Franchat as a potent compound for ameliorating the oxidative organ injury of MTX. According to the predicted target profiles of TMP, a possible mechanism of the abrogation of MTX-induced toxicity is that TMP could upregulate cAMP by inhibiting phosphodiesterase (PDE) 10A2 activity. Another novel finding is that the competitive binding and antagonistic effects of TMP on adenosine receptor 2A and 2B appear to play important roles in the TMP-mediated reversal of MTX-induced hepatic injury. **CONCLUSION:** TMP identified by a network pharmacology approach could ameliorate MTX-induced oxidative organ injury. This study provides important evidence for the preclinical evaluation of TMP and MTX as a novel combinatorial remedy. As taken from Zhang B et al. 2015. J. Ethnopharmacol. 175, 638-47. PubMed, 2016

“Blood-retinal barrier (BRB) breakdown is one of the primary causes of diabetic retinopathy (DR). The pro-inflammatory factor interleukin-1 β (IL-1 β) was reported to be involved in the induction of BRB breakdown during the pathogenesis of DR. In the present study, we investigated the protective effect of tetramethylpyrazine (TMP), a major active component of the traditional herb *Ligusticum chuanxiong*, on IL-1 β -induced cell death of the rat retinal capillary endothelial TR-iBRB2 cells. Our results showed that IL-1 β -induced cell dysfunction in TR-iBRB2 cells via inducing nitrate/oxidative stress; however, such effect was attenuated with the pre-treatment of TMP. The cellular protective effect of TMP was likely to be mediated through the inhibition of inducible nitric oxide synthase (iNOS) expression and leukostasis as well as suppression of reactive oxygen species (ROS) generation, mitochondrial dysfunction and MAPKs activation. These findings significantly contribute to a better understanding of the protective effect of TMP in DR and form the basis of the therapeutic development of TMP in treating such disease in the future.” As taken from Zhu X et al. 2015. Int. J. Mol. Sci. 16(9), 21775-90. PubMed, 2016

“Our recent study demonstrated that sodium arsenite at a clinically relevant dose induced nephrotoxicity in human renal proximal tubular epithelial cell line HK-2, which could be inhibited by natural product 2,3,5,6-tetramethylpyrazine (TMP) with antioxidant activity. The present study demonstrated that arsenic exposure resulted in protein and enzymatic induction of heme oxygenase-1 (HO-1) in dose- and time-dependent manners in HK-2 cells. Blocking HO-1 enzymatic activity by zinc protoporphyrin (ZnPP) augmented arsenic-induced apoptosis, ROS production and mitochondrial dysfunction, suggesting a critical role for HO-1 as a renal protectant in this procession. On the other hand, TMP, upstream of HO-1, inhibited arsenic-induced ROS production and ROS-dependent HO-1 expression. TMP also prevented mitochondria dysfunction and suppressed activation of the intrinsic apoptotic pathway in HK-2 cells. Our results revealed that the regulation of arsenic-induced HO-1 expression was performed through multiple ROS-dependent signal pathways and the corresponding transcription factors, including p38 MAPK and JNK (but not ERK), AP-1, Nrf2 and NF- κ B. TMP inhibited arsenic-induced activations of JNK, p38 MAPK, ERK,

AP-1 and Nrf2 and block HO-1 protein expression. The present study, furthermore, demonstrated arsenic-induced expression of arsenic response protein 2 (ARS2) that was regulated by p38 MAPK, ERK and NF- κ B. To our knowledge, this is the first report showing that ARS2 involved in arsenic-induced nephrotoxicity, while TMP pretreatment prevented such an up-regulation of ARS2 in HK-2 cells. Given ARS2 and HO-1 sharing the similar regulation mechanism, we speculated that ARS2 might also mediate cell survival in this procession. In summary, our study highlighted a role of HO-1 in the protection against arsenic-induced cytotoxicity downstream from the primary targets of TMP and further indicated that TMP may be used as a potential therapeutic agent in the treatment of arsenic-induced nephrotoxicity." As taken from Gong X et al. 2016. Arch. Toxicol. 90(9), 2187-200. PubMed, 2016

"Although kidney is a target organ of arsenic cytotoxicity, the underlying mechanisms of arsenic-induced nephrotoxicity remain poorly understood. As tetramethylpyrazine (TMP) has recently been found to be a renal protectant in multiple kidney injuries, we hypothesize that TMP could suppress arsenic nephrotoxicity. In this study, human renal proximal tubular epithelial cell line HK-2 was used to elucidate the precise mechanisms of arsenic nephrotoxicity as well as the protective mechanism of TMP in these cells. Sodium arsenite exposure dramatically increased cellular reactive oxygen species (ROS) production, decreased levels of cellular glutathione (GSH), decreased cytochrome c oxidase activity and mitochondrial membrane potential, which indicated mitochondrial dysfunction. On the other hand, sodium arsenite activated pro-inflammatory signals, including β -catenin, nuclear factor- κ B (NF- κ B), p38 mitogen-activated protein kinase (MAPK), tumor necrosis factor alpha and cyclooxygenase-2 (COX-2). Small molecule inhibitors of NF- κ B and p38 MAPK blocked arsenic-induced COX-2 expression, suggesting arsenic-induced COX-2 up-regulation was NF- κ B- and p38 MAPK-dependent. Finally, sodium arsenite induced autophagy in HK-2 cells at early phase (6 h) and the subsequent apoptosis at 24 h. Treatment by TMP or by the antioxidant N-acetylcysteine decreased arsenic-induced ROS production, enhanced GSH levels, prevented mitochondria dysfunction and suppressed the activation of pro-inflammatory signals and the development of autophagy and apoptosis. Our results suggested that TMP may be used as a new potential therapeutic agent to prevent arsenic-induced nephrotoxicity by suppressing these pathological processes." As taken from Gong X et al. 2015. Arch. Toxicol. 89(7), 1057-70. PubMed, 2016

"Micro-RNAs (miRNAs) are involved in regulation of the incidence and development of several hepatic diseases. Thus manipulating miRNAs may be a promising therapeutic strategy against these entities. In this study hepatic stellate cells (HSCs) were transfected with hsa-miR-9 or anti-hsa-miR-9, treated with tetramethylpyrazine (TMP), or subjected to treatment with TMP and hsa-miR-9 transfection (combined treatment group). Then, real-time polymerase chain reaction (PCR) was performed to measure mRNA levels of hsa-miR-9. Expression of hsa-miR-9 was highest in the combination treatment group compared with other groups, and significantly higher than TMP-treated and hsa-miR-9-transfected groups (both $P < 0.05$). The anti-hsa-miR-9-transfected group expressed the lowest mRNA level of hsa-miR-9 with marked decrease versus control ($P < 0.05$). Downstream factors that may be affected by miR-9 such as leptin, α -smooth muscle actin (SMA), and collagen I, as well as phosphorylation levels of Janus kinase 1 (JAK1)/signal transducer and activator of transcription 3 (STAT3) were investigated at the protein level. All these factors were regulated contrariwise to expression trends of hsa-miR-9, showing the lowest level in the combination treatment group and highest level in anti-hsa-miR-9-transfected group. These results suggest that both transfection of hsa-miR-9 and TMP can lead to upregulated endogenous expression of hsa-miR-9, inhibit activation of JAK1/STAT3 signal pathway induced by leptin, and lead to reduction of α -SMA and collagen I-thus impeding activation of HSC." As taken from Li SG et al. 2015. Biol. Pharm. Bull. 38(3), 396-401. PubMed, 2016

"Tetramethylpyrazine (TMP), one of the active ingredients isolated from a Chinese herbal prescription, possesses protective effects against apoptosis in endothelial cells. However, the underlying mechanism of its protective effects in endothelial cells remains to be elucidated. Using human umbilical vein endothelial cells (HUVECs), the present study assessed the protective effects of TMP on CoCl₂-induced apoptosis. Following pre-incubation with CoCl₂ (150 μ M/ml) for 4 h, the

HUVECs were treated with TMP at different concentrations (50, 100 and 200 $\mu\text{M}/\text{ml}$) for 8 h. TMP upregulated the expression of prolyl hydroxylase (PHD)2, reduced the protein and mRNA expression levels of vascular endothelial growth factor (VEGF), and reduced the expression of HIF-1 α only at the protein level, not at the mRNA level in HUVECs, in a concentration-dependent manner. Furthermore, silencing of the PHD2 gene with small interfering (si)RNAs abolished the reduction in the expression of hypoxia-inducible factor (HIF)-1 α and VEGF by TMP. In addition, TMP protected CoCl₂-induced HUVEC injury via an apoptosis pathway, as characterized by the increased ratio of cell viability and the reduced percentage of apoptotic and terminal deoxynucleotidyl transferase dUTP nick end labeling-positive HUVECs, activation of caspase-3, -8 and -9, B-cell lymphoma (Bcl)-2/Bcl-2-activated X protein expression, as well as the release of cytochrome c. The protective properties of TMP were partially attributed to the mRNA and protein expression levels of PHD, since silencing of the PHD2 gene with siRNAs abolished these effects. The present study demonstrated that the antiapoptotic effect of TMP in CoCl₂-induced HUVECs was, at least in part, via the regulation of the PHD2/HIF-1 α signaling pathway.” As taken from Yang C et al. 2016. *Mol. Med. Rep.* 13(2), 1287-96. PubMed 2016

“To study the effects of tetramethylpyrazine (TMP) on the proliferation of hepatic stellate cells-T6 (HSC-T6), and the expression of connective tissue growth factor (CTGF) and Smad2/3 in these cells, HSC-T6 cells were cultured with TMP at different concentrations after transforming growth factor- β 1 (TGF- β 1) stimulation. MTT assay was used to assess the cell proliferation. Cells were divided into the control group, TGF- β 1-treated group and TMP-treated groups, which were treated with different concentrations of TMP. Immunocytochemistry and western blot were performed to detect the expression levels of CTGF and Smad2/3 in HSC-T6 cells. MTT analysis indicated that TMP significantly inhibited the proliferation of HSC-T6 cells, in dose-dependent and time-dependent manners. Immunocytochemistry detection and western blot showed that TMP could diminish TGF- β 1-induced CTGF over-expression in HSC-T6 cells. Similarly, the enhancing effects of TGF- β 1 on Smad2/3 expressions in HSC-T6 cells could also be counteracted by TMP treatment. Nuclear translocation of Smad2/3 was blocked by TMP treatment. Correlation analysis suggested a positive correlation between CTGF and Smad2/3 expression levels in HSC-T6 cells. TMP exerts anti-hepatic fibrosis effect through decreasing the expression of CTGF and Smad2/3, as well as inhibiting the proliferation of HSC-T6 cells. Our study provides cellular and molecular bases for further application of TMP in the clinical treatment for hepatic fibrosis.” As taken from Li J et al. 2015. *Biotechnol. Biotechnol. Equip.* 29(1), 124-131. PubMed, 2016

“BACKGROUND AND AIM: Tetramethylpyrazine (TMP), a major alkaloid isolated from *Ligusticum chuanxiong*, has been reported in hepatic fibrosis models. However, the action mechanism remains unclear. In the present study, effects of tetramethylpyrazine (TMP) against hepatic stellate cell (HSC) activation as well as the possible mechanisms were evaluated. METHODS: Western blot assay was used to detect TMP effects on protein expression of Smo, Patched, Hhip, and Gli and to investigate the effects of TMP on Cyclin D1, Cyclin E1, CDK2, Bcl-2, Bax, and caspase expression with cyclopamine supplementation. RESULTS: Our results showed that TMP significantly inhibits the expression of Cyclin D1, Cyclin E1, and Cyclin-dependent kinase CDK2 and changes the HSC cycle by inhibiting the proliferation of HSC. Moreover, TMP has also been shown to decrease the expression of Bcl-2 and increase the expression of Bax in HSC-T6 cells. Furthermore, TMP can inhibit the expression of connective tissue growth factor (CTGF), and the inhibitory effect was intensified after the application of joint treatment with TMP and cyclopamine. CONCLUSION: TMP may be an effective Hh signaling pathway inhibitor for hepatic fibrosis treatment.” As taken from Hu J et al. 2015a. *Biomed. Res. Int.* 2015, 603067. PubMed, 2016

“BACKGROUND: A traditional Chinese medicine, Tetramethylpyrazine (TMP), has been prescribed as a complementary treatment for glaucoma to improve patient prognosis. However, the pharmacological mechanism of action of TMP is poorly understood. In previous studies, we demonstrated that TMP exerts potent inhibitory effects on neovascularization, suppresses the tumorigenic behavior of glioma cells, and protects neural cells by regulating CXCR4 expression. Here, we further investigated whether the SDF-1/CXCR4 pathway is also involved in the TMP-

mediated activity in trabecular meshwork cells. METHODOLOGY/PRINCIPAL FINDINGS: CXCR4 expression was examined by quantitative real-time PCR in trabecular and iris specimens from 54 primary open-angle glaucoma (POAG) patients who required surgery and 19 non-glaucomatous donors. Our data revealed markedly elevated CXCR4 expression in the trabecular meshwork of POAG patients compared with that of controls. Consistently, CXCR4 expression was much higher in glaucomatous trabecular meshwork cells than in normal trabecular meshwork cells. Using RT-PCR and western blot assays, we determined that glaucoma-related cytokines and dexamethasone (DEX) also significantly up-regulated CXCR4 expression in primary human trabecular meshwork (PHTM) cells. Moreover, the TGF- β 1-mediated induction of CXCR4 expression in PHTM cells was markedly down-regulated by TMP compared with control treatment (PBS) and the CXCR4 antagonist AMD3100. In addition, TMP could counteract the TGF- β 1-induced effects on stress fiber accumulation and expansion of PHTM cells. TMP markedly suppressed the migration of PHTM cells stimulated by TGF- β 1 in transwell and scratch wound assays. TMP also suppressed the extracellular matrix (ECM) accumulation induced by TGF- β 2. CONCLUSIONS: Our findings demonstrate that CXCR4 might be involved in the pathogenetic changes in the trabecular meshwork of patients with POAG. Additionally, TMP might exert its beneficial effects in POAG patients by down-regulating CXCR4 expression.” As taken from Yu N et al. 2015. PLoS One 10(8), e0133055. PubMed, 2016

“The present study evaluated the ability and optimal concentration of tetramethylpyrazine (TMP) to induce human umbilical cord-derived mesenchymal stem cells (hUMSCs) to differentiate into neuron-like cells in vitro. Human umbilical cords from full-term caesarean section patients were used to obtain hUMSCs by collagenase digestion after removal of the umbilical artery and vein. The surface antigen expression profile of cultured hUMSCs was monitored by flow cytometry. After amplification, cells of the 5th passage were divided into experimental groups A-C treated with TMP at 4.67, 2.34 and 1.17 mg/ml, respectively, in low glucose-Dulbecco's Modified Eagle's Medium (L-DMEM) (induction medium), while group D (control) was exposed to L-DMEM culture medium only. Differentiation of hUMSCs into neuron-like cells and morphological changes were observed every 0.5 h with an inverted phase contrast microscope for 6 h. After the 6-h induction period, proportions of cells expressing neuronal markers neuron-specific enolase (NSE), neurofilament protein (NF-H) and glial fibrillary acidic protein (GFAP) were detected by immunohistochemistry. The optimal concentration of TMP was selected on the basis of neuron-like cell positive rate. Western blotting and RT-polymerase chain reaction were applied to detect the expression of NSE, NF-H, and GFAP of the group of optimal concentration in each point-in-time. Results showed that most primary cells were adherent 12 h after seeding and first appeared as diamond or polygon shapes. Thereafter, they gradually grew into long spindle-shaped cells and finally in a radiating or swirling pattern. The cells maintained a strong proliferative capacity after continuous passage. Flow cytometry analysis of cultured hUMSCs at the 3rd, 5th and 10th passages expressed CD73, CD90 and CD105, but not CD11b, CD19, CD34, CD45 or human leukocyte antigen-DR. After 6 h of TMP treatment, typical neuron-like cells with many protrusions connected into a net-like pattern were observed in all experimental groups. These neuron-like cells were positive for NSE and NF-H, but negative for GFAP. Among the tested treatment groups, group A with TMP at 4.67 mg/ml had the highest expression of NSE and NF-H. By contrast, no change was found after induction in the control group. The mRNA expression of cells expressing neuronal markers as well as GAPDH was observed, with the relative NSE transcript levels of 0, 1.303 ± 0.031 , 1.558 ± 0.025 , 1.927 ± 0.019 and 2.415 ± 0.033 after 0, 1, 2, 4 and 6 h of treatment, respectively; the mRNA expression of NF-H was 0, 1.429 ± 0.025 , 1.551 ± 0.024 , 1.930 ± 0.042 and 1.398 ± 0.014 after 0, 1, 2, 4 and 6 h of treatment, respectively. There was no expression of GFAP before or after induction and all the groups showed high expression of GAPDH at each time point. Protein expression was also observed on cells expressing neuronal markers as well as GAPDH at each time point. The protein expression of NSE was 0, 0.717 ± 0.097 , 0.919 ± 0.056 , 1.097 ± 0.143 and 1.157 ± 0.055 in proper order; the protein expression of NF-H was 0, 0.780 ± 0.103 , 0.973 ± 0.150 , 1.053 ± 0.107 and 0.753 ± 0.094 in proper order. There was no expression of GFAP before or after induction, and all the groups showed high expression of GAPDH at each tested time point. Our results demonstrated that TMP can induce

hUMSCs to differentiate into neuron-like cells effectively with the optimal concentration of 4.67 mg/ml. After induction, the NSE and NF-H of the neuron-like cells were positive, but the GFAP-2 was negative.” As taken from Nan C et al. 2017. *Int. J. Oncol.* 48(6), 2287-94. PubMed, 2018

“Aging drives the accumulation of senescent cells (SnCs) including stem/progenitor cells in bone marrow, which contributes to aging-related bone degenerative pathologies. Local elimination of SnCs has been shown as potential treatment for degenerative diseases. As LepR⁺ mesenchymal stem/progenitor cells (MSPCs) in bone marrow are the major population for forming bone/cartilage and maintaining HSCs niche, whether local elimination of senescent LepR⁺ MSPCs delays aging-related pathologies and improves local microenvironment need to be well defined. In this study, we performed local delivery of tetramethylpyrazine (TMP) in bone marrow of aging mice, which previously showed to be used for the prevention and treatment of glucocorticoid-induced osteoporosis (GIOP). We found the increased accumulation of senescent LepR⁺ MSPCs in bone marrow of aging mice, and TMP significantly inhibited the cell senescent phenotype via modulating Ezh2-H3k27me3. Most importantly, local delivery of TMP improved bone marrow microenvironment and maintained bone homeostasis in aging mice by increasing metabolic and anti-inflammatory responses, inducing H-type vessel formation, and maintaining HSCs niche. These findings provide evidence on the mechanisms, characteristics and functions of local elimination of SnCs in bone marrow, as well as the use of TMP as a potential treatment to ameliorate human age-related skeletal diseases and to promote healthy lifespan.” As taken from Gao B et al. 2018. *Aging Cell.* 17(3), e12741. PubMed, 2019

“Accumulating evidence indicates that hedgehog signaling plays a pivotal role in pathological angiogenesis and is involved in wound-healing responses in a number of adult tissues, including the liver. We previously demonstrated that hedgehog signaling promoted proliferation and inhibited apoptosis in hepatic stellate cells. This study was aimed to evaluate the effect of tetramethylpyrazine (TMP) on hedgehog signaling and to further examine the molecular mechanisms of TMP-induced antiangiogenic effects in liver fibrosis. We found that TMP ameliorated the expression of proangiogenic markers vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor receptor 2 (VEGF-R2), platelet-derived growth factor BB (PDGF-BB), platelet-derived growth factor- β receptor (PDGF- β R) and hypoxia inducible factor 1 α (HIF-1 α), concomitant with reduced abundance of endothelial markers platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), CD34 and von willebrand factor in vivo and in vitro. Interestingly, TMP attenuated the abundance of sonic hedgehog, smoothened (Smo) and glioblastoma but increased the expression of hedgehog-interacting protein in liver sinusoidal endothelial cells, which was underlying mechanism for the antiangiogenic activity of TMP. Downregulation of Smo activity, using selective Smo inhibitor cyclopamine, lead to a synergistic effect with TMP, whereas Smo overexpression plasmid impaired the induction of antiangiogenic effects of TMP. Overall, these results provide novel implications to reveal the molecular mechanism of TMP-inhibited liver sinusoidal angiogenesis, by which points to the possibility of using TMP-based antiangiogenic drugs for the treatment of liver fibrosis.” As taken from Zhao S et al. 2017. *IUBMB Life* 69(2), 115-127. PubMed, 2018

“Accumulating evidence indicates that hepatic stellate cells (HSCs) are the central mediators and major effectors in the development of hepatic fibrosis. It is well-known that regulation of cell proliferation and apoptosis are potential strategies to block the activation of HSCs. Recently, several studies have revealed that induction of HSC senescence could prevent and cure the liver fibrosis. In our previous work, we have demonstrated that the natural product tetramethylpyrazine (TMP) could inhibit the activation of HSCs and ameliorate hepatic fibrosis. The aim of this study was to identify a new role of TMP in the regulation of activated HSC senescence and to elucidate the underlying mechanisms. In this study, our data showed that TMP could promote HSC senescence in vivo and in vitro. Moreover, TMP affected the cell cycle and telomerase activity. We further demonstrated that P53 siRNA or P53 pharmacological inhibitor PFT- α abrogated the TMP-induced HSC senescence in vitro. Meanwhile, similar results were obtained in vivo. Further studies indicated that TMP promoted the expression of P53 through a YAP inhibition-dependent

mechanism. Moreover, silencing YAP enhanced TMP induction of activated HSC senescence. Collectively, our results suggested that TMP inhibited the activation of HSCs by inducing senescence and had therapeutic implication for the treatment of liver fibrosis.” As taken from Jin H et al. 2017. *Eur. J. Pharm. Sci.* 96, 323-333. PubMed, 2018

“Objective To investigate the effects of tetramethylpyrazine (TMP) on experimental autoimmune myasthenia gravis (EAMG) in rats and explore the possible immune regulation mechanism. Methods Lewis rats were randomly divided into 4 groups: control group, EAMG group, TMP low-dose group (TMP-L, 10 mg/kg) and TMP high-dose group (TMP-H, 20 mg/kg). Except the control group, the other 3 groups were subjected to EAMG modeling. The body mass was determined and the symptoms of muscular weakness in rats were scored by Lennon EAMG criteria. The expressions of acetylcholine receptor (AChR), IgG and C3 in neuromuscular junctions were examined by immunofluorescence. The levels of R97-116 antibody (IgG1, IgG2a, IgG2b), tumor necrosis factor α (TNF- α), interferon γ (IFN- γ) and interleukin 10 (IL-10) were detected by ELISA. Results TMP decreased the scores in the evaluation of the symptoms of muscle weakness in EAMG rats. Moreover, TMP administration significantly inhibited AChR expression and increased IgG and C3 expression in neuromuscular junctions of EAMG rats. In addition, after TMP treatment, the levels of IgG1, IgG2a and TNF- α were remarkably reduced, while IL-10 level was elevated. However, there was no significant change in serum levels of IgG2b and IFN- γ . Conclusion TMP can exert inhibitory effects on Th1, Th2 and B cells and modulate the levels of IgG1, IgG2a, TNF- α and IL-10. All these effects contribute to relieve the symptoms of muscle weakness in EAMG rats.” As taken from Ren Y et al. 2016. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 32(11), 1471-1474. PubMed, 2018

“Rationale: Hepatic stellate cells (HSCs) are liver-specific pericytes regulating vascular remodeling during hepatic fibrosis. Here, we investigated how ligustrazine affects HSC pericyte functions. Methods: Rat HSC-T6 and human HSC-LX2 cells were cultured, and multiple molecular experiments including real-time PCR, Western blot, flow cytometry, immunofluorescence, electrophoretic mobility shift assay and co-immunoprecipitation were used to elucidate the underlying mechanisms. Molecular simulation and site-directed mutagenesis were performed to uncover the target molecule of ligustrazine. Rats were intoxicated with CCl₄ for evaluating ligustrazine's effects in vivo. Results: Ligustrazine inhibited angiogenic cytokine production, migration, adhesion and contraction in HSCs, and activated PPAR γ . Selective PPAR γ inhibitor GW9662 potentially abrogated ligustrazine suppression of HSC pericyte functions. Additionally, HIF-1 α inhibitor PX-478 repressed HSC pericyte functions, and ligustrazine inhibited the transcription of HIF-1 α , which was diminished by GW9662. Moreover, ligustrazine downregulation of HIF-1 α was rescued by knockdown of SMRT, and ligustrazine increased PPAR γ physical interaction with SMRT, which was abolished by GW9662. These findings collectively indicated that activation of PPAR γ by ligustrazine led to transrepression of HIF-1 α via a SMRT-dependent mechanism. Furthermore, molecular docking evidence revealed that ligustrazine bound to PPAR γ in a unique double-molecule manner via hydrogen bonding with the residues Ser289 and Ser342. Site-directed mutation of Ser289 and/or Ser342 resulted in the loss of ligustrazine transrepression of HIF-1 α in HSCs, indicating that interactions with both the residues were indispensable for ligustrazine effects. Finally, ligustrazine improved hepatic injury, angiogenesis and vascular remodeling in CCl₄-induced liver fibrosis in rats. Conclusions: We discovered a novel ligand activation pattern for PPAR γ transrepression of the target gene with therapeutic implications in HSC pericyte biology and liver fibrosis.” As taken from Zhang F et al. 2018. *Theranostics* 8(3), 610-626. PubMed, 2018

“Tetramethylpyrazine is a bioactive compound found in Chinese black vinegar. This work is to investigate the potential effects of tetramethylpyrazine on intracellular cholesterol modulation in HepG2 cells. The results demonstrated that tetramethylpyrazine can induce intracellular cholesterol efflux. Tetramethylpyrazine may also improve endothelial function through its antioxidant effects by inhibiting reactive oxygen species levels and increasing the antioxidant enzymes superoxide dismutase and catalase. Tetramethylpyrazine increased liver X receptor and peroxisome proliferator-activated receptor gene expression in HepG2 cells. Protein expression of ATP-binding

cassette transporter 1 was up-regulated in a dose-dependent manner ($P < 0.05$). The biological significance of tetramethylpyrazine may involve hypolipidemic effects via modulation of intracellular cholesterol efflux, ROS inhibition, increases in SOD and CAT activities, and direct regulation of PPAR and LXR gene expression. Tetramethylpyrazine may improve lipid profiles by elevating the PPAR γ -LXR α -ABCA1 pathway." As taken from Chen J et al. 2017. Food Chem. Toxicol. 109(Pt. 2), 930-940. PubMed, 2018

"Tetramethylpyrazine (TMP) is a compound purified from herb. Its effect on Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) in renal cells is unclear. This study examined whether TMP altered Ca^{2+} signaling in Madin-Darby canine kidney (MDCK) cells. TMP at 100-800 μM induced $[\text{Ca}^{2+}]_i$ rises, which were reduced by Ca^{2+} removal. TMP induced Mn^{2+} influx implicating Ca^{2+} entry. TMP-induced Ca^{2+} entry was inhibited by 30% by modulators of protein kinase C (PKC) and store-operated Ca^{2+} channels. Treatment with the endoplasmic reticulum Ca^{2+} pump inhibitor 2,5-di-tert-butylhydroquinone (BHQ) inhibited 93% of TMP-evoked $[\text{Ca}^{2+}]_i$ rises. Treatment with TMP abolished BHQ-evoked $[\text{Ca}^{2+}]_i$ rises. Inhibition of phospholipase C (PLC) abolished TMP-induced responses. TMP at 200-1000 μM decreased viability, which was not reversed by pretreatment with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester. Together, in MDCK cells, TMP induced $[\text{Ca}^{2+}]_i$ rises by evoking PLC-dependent Ca^{2+} release from endoplasmic reticulum and Ca^{2+} entry via PKC-sensitive store-operated Ca^{2+} entry. TMP also caused Ca^{2+} -independent cell death." As taken from Fang YC et al. 2017. J. Biochem. Mol. Toxicol. 31(10), e21952. PubMed, 2018

"BACKGROUND: Transplantation of bone marrow-derived mesenchymal stem cells (BMSCs) is one of the new therapeutic strategies for treating ischemic stroke. However, the relatively poor migratory capacity of BMSCs toward infarcted regions limited the therapeutic potential of this approach. Pharmacological preconditioning can increase the expression of CXC chemokine receptor 4 (CXCR4) in BMSCs and enhance cell migration toward the injury site. In the present study, we investigated whether tetramethylpyrazine (TMP) preconditioning could enhance BMSCs migration to the ischemic brain and improve functional recovery through upregulating CXCR4 expression. METHODS: BMSCs were identified by flow cytometry analysis. BMSCs migration was evaluated in vitro by transwell migration assay, and CXCR4 expression was measured by quantitative reverse transcription-polymerase chain reaction and western blot analysis. In rats with focal cerebral ischemia, the neurological function was evaluated by the modified neurological severity score, the adhesive removal test and the corner test. The homing BMSCs and angiogenesis were detected by immunofluorescence, and expression of stromal cell-derived factor-1 (SDF-1) and CXCR4 was measured by western blot analysis. RESULTS: Flow cytometry analysis demonstrated that BMSCs expressed CD29 and CD90, but not CD34 and CD45. TMP pretreatment dose-dependently induced BMSCs migration and CXCR4 expression in vitro, which was significantly inhibited by AMD3100, a CXCR4 antagonist. In rat stroke models, we found more TMP-preconditioned BMSCs homing toward the infarcted regions than nonpreconditioned cells, leading to improved neurological performance and enhanced angiogenesis. Moreover, TMP-preconditioned BMSCs significantly upregulated the protein expression of SDF-1 and CXCR4 in the ischemic boundary regions. These beneficial effects of TMP preconditioning were blocked by AMD3100. CONCLUSION: TMP preconditioning enhances the migration and homing ability of BMSCs, increases CXCR4 expression, promotes angiogenesis, and improves neurological performance. Therefore, TMP preconditioning may be an effective strategy to improve the therapeutic potency of BMSCs for ischemic stroke due to enhanced BMSCs migration to ischemic regions." As taken from Li L et al. 2017. Stem Cell Res. Ther. 8(1), 112. PubMed, 2018

"The natural small molecule compound: 2,3,5,6-tetramethylpyrazine (TMP), is a major component of the Chinese medicine Chuanxiong, which has wide clinical applications in dilating blood vessels, inhibiting platelet aggregation and treating thrombosis. Recent work suggests that TMP is also an antitumour agent. Despite its chemotherapeutic potential, the mechanism(s) underlying TMP action are unknown. Herein, we demonstrate that TMP binds to mitochondrial transcription factor A (TFAM) and blocks its degradation by the mitochondrial Lon protease. TFAM is a key regulator of

mtDNA replication, transcription and transmission. Our previous work showed that when TFAM is not bound to DNA, it is rapidly degraded by the ATP-dependent Lon protease, which is essential for mitochondrial proteostasis. In cultured cells, TMP specifically blocks Lon-mediated degradation of TFAM, leading to TFAM accumulation and subsequent up-regulation of mtDNA content in cells with substantially low levels of mtDNA. In vitro protease assays show that TMP does not directly inhibit mitochondrial Lon, rather interacts with TFAM and blocks degradation. Pull-down assays show that biotinylated TMP interacts with TFAM. These findings suggest a novel mechanism whereby TMP stabilizes TFAM and confers resistance to Lon-mediated degradation, thereby promoting mtDNA up-regulation in cells with low mtDNA content.” As taken from Lan L et al. 2017. *Biosci. Rep.* 37(3), BSR20170319. PubMed, 2018

“Tetramethylpyrazine (TMP) has exhibited various anticancer effects. However, its ability to inhibit proliferation, migration, and invasion of prostate cancer (PCa) PC-3 cells is still unclear. In the present study, different concentrations of TMP were co-incubated with PC-3 cells. The pcDNA-FOXM1 plasmid was transfected into cells before treatment with 500 µg/l TMP. The proliferative, migratory and invasive abilities of PC-3 cells were tested by MTT assay, wound healing assay and colony formation assay. Western blotting was used to investigate the expression of FOXM1. We found that, compared with the control, the proliferative, migratory and invasive abilities of PC-3 cells were decreased after incubation with different concentrations of TMP ($P<0.01$). The expression of FOXM1 was decreased in TMP-treated PC-3 cells ($P<0.01$). In addition, overexpression of FOXM1 reversed TMP-mediated inhibition of proliferation, migration and invasion of PC-3 cells. We also found that TMP inhibited PCa growth in vivo in a dose-dependent manner. These results suggest that TMP inhibits PC-3 cell proliferation, migration and invasion by downregulation of FOXM1.” As taken from Zhou Y et al. 2017. *Oncol. Rep.* 38(2), 837-842. PubMed, 2018

“Retinoblastoma is the most common ocular tumor in children, and it causes extensive damage. Current treatment options for retinoblastoma include surgery, chemotherapy, radiotherapy and cryotherapy. However, the majority of chemotherapy medicines cause complications and side effects that lead to severe impairment of patient health. Previous studies have reported that tetramethylpyrazine (TMP), which is an extract of the Chinese herbal medicine Chuanxiong, reduces the risk of multidrug resistance in chemotherapy and inhibits the proliferation and metastasis of various types of cancer cells. However, the underlying molecular mechanism of TMP in retinoblastoma remains unclear. The current study demonstrated that C-X-C chemokine receptor type 4 (CXCR4) was expressed in WERI-Rb1 cells and in retinoblastoma. Using reverse transcription-quantitative polymerase chain reaction and western blotting techniques, the current study demonstrated that TMP significantly downregulated the expression of CXCR4 in WERI-Rb1 cells cultured at high density, whereas it had a minor effect in low-density WERI-Rb1 cells; additionally, this effect occurred in a time-dependent manner. TMP inhibited the proliferation of WERI-Rb1 cells as effectively as a CXCR4 antagonist, AMD3100, consistent with a role of CXCR4 in cancer development. Notably, TMP did not affect the cell cycle of cells cultured at low density (1×10^5 cells/ml), whereas it induced G1-phase arrest in high-density cells (7.5×10^5 cells/ml; $P<0.05$). In addition, the expression of CXCR4 in primary rat retinal neurocytes was significantly downregulated by TMP treatment, and this treatment protected primary rat retinal neurocytes from H₂O₂-induced damage. Thus, the results of this study indicate that TMP is a potential candidate for use in treatment of retinoblastoma, and also provides novel insights into the mechanisms of the anti-cancer and neuroprotective effects of this extract.” As taken from Wu N et al. 2017. *Mol. Med. Rep.* 15(5), 2481-2488. PubMed, 2018

TMP, an alkaloid derived from *Ligusticum sinense* ‘Chuanxiong’, is renowned for its protective role against kidney injuries caused by gentamicin, diabetes mellitus, and ischemia–reperfusion injury. Our team’s prior research has shown that TMP counters CM-induced renal injury by inhibiting p38 MAPK activation. Further investigations revealed TMP’s capacity to reverse CM-induced activation of the CCL2/CCR2 pathway, ameliorate renal oxidative stress and abnormal mitochondrial dynamics, and regulate mitophagy in tubular cells, underscoring TMP’s renoprotective effects. As taken from Wang Z et. Al. 2024 *Toxics*.

Pioglitazone (Piog), a thiazolidinedione, and ligustrazine (Ligu), a natural component of *Ligusticum chuanxiong* possess gastroprotective properties. However, the underlying mechanism is not well elucidated. The present study aimed to investigate the gastroprotective effects of Piog (15 mg/kg, p.o.), Ligu (15 mg/kg, p.o.), and their combination against ethanol-induced gastric ulcer in rats. Omeprazole (10 mg/kg) was used as a standard. Pre-treatment for 7 days with Piog, Ligu, and (Piog+Ligu) effectively alleviated ethanol-predisposed oxidative stress and inflammation through restoring HO-1, GSH, and SOD tissue levels and decreasing elevated MDA, TNF- α , ICAM, i-NOS, and IL-1 β contents. Moreover, Piog, Ligu, and (Piog+Ligu) markedly inhibited the ethanol-induced increase of gastric NF-KB and BAX. In contrast, this pre-treatment regimen significantly accelerated protein expression of SIRT1, Nrf2, and Bcl-2, along with autophagic proteins, ATG5 and Beclin. Interestingly, macroscopic, histopathological examination and mucin content were in harmony with previous results, where pre-treatment with Piog, Ligu, and (Piog+Ligu) showed a declined mucosal injury as evidenced by the remarkable decrease of the ulcer area percentage by 62.3%, 38.7%, and 91.2%, respectively, compared to the ethanol-ulcerated group. In conclusion, Piog and Ligu exhibited remarkable gastroprotective properties. Our study was the first to show that Piog, Ligu, and (Piog+Ligu) ameliorated oxidative stress, inflammation, and apoptosis and accelerated the autophagic process via the upregulation of the upstream SIRT1 protein. As taken from Mahmoud SA et. Al. 2024. Naunyn Schmiedeberg's Arch Pharmacol.

Based on network pharmacology, molecular docking simulation and animal experiments, this study explored the target and molecular mechanism of TMP in the treatment of OA.(...) Animal models of rabbit knee osteoarthritis were prepared, and magnetic resonance imager (MRI) and fluorescence quantitative PCR (RT-qPCR) were used to observe the effect of TMP in treating OA as well as the expression of key target genes. (...) Animal experiments showed that TMP had a significant therapeutic effect on OA. The TMP group had significantly reduced knee joint effusion and bone marrow damage compared to the OA group ($p < 0.05$). (...)Conclusion: TMP is effective in the treatment of OA, with multi-target and multi-pathway interactions. ESR1, CAT, C3, CYP3A4, CYP2C9, and ANXA1 may be potential targets for TMP treatment of OA. The molecular mechanism mainly involves the formation of neutrophil extracellular trap, regulation of the actin cytoskeleton, complement and coagulation cascades, and T cell receptor signaling pathways, etc. As taken from Li J et. Al. 2024. Front Chem.

In this study, TMP was tested for its specific activities in rat aortic endothelial cells (RAECs) and fractured rat model.Methods: The effect of TMP on angiogenesis and migration in RAECs was detected by conducting matrigel tubulogenesis assay and transwell assay. Histopathological changes were observed in the rats from each group using H&E staining. The levels of inflammation and coagulation markers in rats were evaluated by ELISA. The expression of osteogenesis-related genes in rats was assessed by RT-qPCR and western blotting.Results: TMP promoted angiogenesis processes and migratory ability in RAECs. TMP improved histopathological changes in fractured rat model. The concentration of inflammatory markers (IL-2, IL-6, IL-1 β) in the serum of fractured rats were suppressed by TMP treatment. TMP also had the potential to inhibit blood coagulation in rat tibia fracture model. In addition, the expression and protein levels of osteogenesis-related markers (ALP, Runx2, and OPN-1) were elevated by TMP in the tissues from the fractured rats. In mechanism, TMP significantly promoted the activation of VEGF/FLK1 pathway in vitro and in vivo.Conclusion: TMP accelerated the repair of bone fracture by promoting angiogenesis and osteogenesis. As taken from Ai J et. Al. 2025. J Orthop Surg Res.

Jiang et al. constructed a rat model to determine the duration of renal I/R on the recovery of RIRI and found that RIRI in rats became more severe at 24 h and gradually recovered with the prolongation of reperfusion time. In this model, TMP could prevent AKI through suppressing inflammation (Jiang et al., 2020). The severity of I/R injury is influenced by various factors, including the duration of ischemia, oxygen demand of organs, reperfusion pressure, oxygen content of the perfusion fluid, as well as temperature. It has been observed that warm I/R can cause more severer damage than cold storage (Feng et al., 2004). Feng et al. revealed that TMP protected mice against renal warm I/R because it suppressed necrosis and apoptosis of renal

tubular epithelial cells and reduced neutrophil infiltration (Feng et al., 2011). In recent years, adriamycin-induced nephropathy in rats has been widely applied in pharmacological research of human chronic kidney disease. Research has shown that TMP had a protective effect on adriamycin-induced NRK-52E, a kind of rat renal tubular cell (Cheng et al., 2006). (...) Recent studies have shown that TMP can alleviate HIRI through suppressing infiltration of immune cells such as neutrophils (Liu et al., 2021). Chen et al. found that administration of TMP ameliorated HIRI and enhanced liver functions 5 min before reperfusion (Chen et al., 2003)..As taken from Qi M et. al. 2024 Pharmacol Ther.**7. Addiction**

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 2,3,5,6-Tetramethylpyrazine 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 2,3,5,6-Tetramethylpyrazine. Table below provides tested level(s) and specific endpoint(s).

Endpoint	Tested level (ppm)	Reference
Smoke chemistry	24	Carmines, 2002 & Rustemeier et al., 2002
	23	Baker et al., 2004a
	1.95 26	JTI KB Study Report(s)
	3,150	Gaworski et al., 2011 & Coggins et al., 2011d
	3	Roemer et al., 2014
In vitro genotoxicity	24	Carmines, 2002 & Roemer et al., 2002
	23	Baker et al., 2004c
	1.95	Renne et al., 2006
	1.95 26	JTI KB Study Report(s)
	3,150	Gaworski et al., 2011 & Coggins et al., 2011d
	3	Roemer et al., 2014
In vitro cytotoxicity	24	Carmines , 2002 & Roemer et al., 2002
	23	Baker et al., 2004c
	1.95 26	JTI KB Study Report(s)
	3,150	Gaworski et al., 2011 & Coggins et al., 2011d
	3	Roemer et al., 2014
Inhalation study	<0.1	Gaworski et al., 1998
	24	Carmines, 2002 & Vanscheeuwijck et al., 2002
	23	Baker et al., 2004c

	1.95	Renne et al., 2006
	1.95 26	JTI KB Study Report(s)
	3	Schramke et al., 2014
Skin painting	<0.1	Gaworski et al., 1999
	1.95 26	JTI KB Study Report(s)
In vivo genotoxicity	3	Schramke et al., 2014

9. Heated/vapor emissions toxicity

Total particulate matter (TPM) from heated (tobacco or nicotine) product(s) containing Tetramethylpyrazine, [2,3,5,6-] 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 Tetramethylpyrazine, [2,3,5,6-] 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	6.5	JTI KB Study Report(s)
In vitro cytotoxicity	6.5	JTI KB Study Report(s)

Aerosol from an electronic nicotine delivery system (ENDS) that creates a vapor by heating an e-liquid containing 2,3,5,6-Tetramethylpyrazine was tested in a battery of in vitro test(s). Under the test conditions and within the sensitivity and specificity of the bioassay(s), no mutagenic, genotoxic or cytotoxic responses were observed when exposed to Aerosol Collected Matter (ACM) and/or aerosol Gas Vapor Phase (GVP) after exposure to the aerosol even when exposure concentrations were the maximal amount that could be achieved with the specific product(s). These results are in contrast to those observed with combustible cigarette which showed mutagenic, genotoxic, cytotoxic responses upon exposure. The table below provides the highest tested level(s) and specific endpoint(s):

Endpoint	Tested level (ppm)	Reference
Aerosol chemistry	150	Labstat International Inc. (2021)
In vitro genotoxicity	150	Labstat International Inc. (2022)
In vitro cytotoxicity	150	Labstat International Inc. (2022)

Aerosol from heated tobacco stick(s) containing 2,3,5,6-Tetramethylpyrazine was tested in aerosol chemistry and a battery of in vitro test(s). Under the test conditions and within the sensitivity and specificity of the bioassay(s), the activity of the total particulate matter (TPM) and/or gas vapor phase (GVP) were not increased by the addition of this ingredient when compared to TPM and/or GVP from reference combustible cigarettes. The table below provides the highest tested level(s) and specific endpoint(s):

Endpoint	Tested level (mg/stick)	Reference
Aerosol chemistry	0.00665	Labstat International Inc. (2021a) Labstat International Inc. (2023a) JTI Heated Tobacco Stick Study Report(s)
In vitro genotoxicity	0.00665	Labstat International Inc. (2021b) Labstat International Inc. (2023b) JTI Heated Tobacco Stick Study Report(s)
In vitro cytotoxicity	0.00665	Labstat International Inc. (2021b) Labstat International Inc. (2023b) JTI Heated Tobacco Stick Study Report(s)

10. Ecotoxicity

10.1. Environmental fate

EPISuite provides the following data:

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

Bond Method :	4.33E-006 atm-m3/mole (4.39E-001 Pa-m3/mole)
Group Method:	2.45E-008 atm-m3/mole (2.48E-003 Pa-m3/mole)
Henrys LC [via VP/WSol estimate using User-Entered or Estimated values]:	HLC: 3.760E-006 atm-m3/mole (3.809E-001 Pa-m3/mole) VP: 0.147 mm Hg (source: MPBPVP) WS: 7.01E+003 mg/L (source: WSKOWWIN)

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

Log Kow used:	1.28 (exp database)
Log Kaw used:	-3.752 (HenryWin est)
Log Koa (KOAWIN v1.10 estimate):	5.032
Log Koa (experimental database):	None

Probability of Rapid Biodegradation (BIOWIN v4.10):

Biowin1 (Linear Model):	0.9014
Biowin2 (Non-Linear Model) :	0.9672
Biowin3 (Ultimate Survey Model):	2.5988 (weeks-months)
Biowin4 (Primary Survey Model) :	3.3771 (days-weeks)
Biowin5 (MITI Linear Model) :	0.4728
Biowin6 (MITI Non-Linear Model):	0.4558
Biowin7 (Anaerobic Linear Model):	-0.6510
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):	71.9 Pa (0.539 mm Hg)
Log Koa (Koawin est):	5.032

Kp (particle/gas partition coef. (m3/ug)):	4.17E-008
Mackay model:	2.64E-008
Octanol/air (Koa) model:	

Fraction sorbed to airborne particulates (phi):

Junge-Pankow model:	1.51E-006
Mackay model:	3.34E-006
Octanol/air (Koa) model:	2.11E-006

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

OVERALL OH Rate Constant =	2.8995 E-12 cm3/molecule-sec
Half-Life =	3.689 Days (12-hr day; 1.5E6 OH/cm3)
Half-Life =	44.266 Hrs
Ozone Reaction:	No Ozone Reaction Estimation
Fraction sorbed to airborne particulates (phi): 2.42E-006 (Junge-Pankow, Mackay avg) 2.11E-006 (Koa method) Note: the sorbed fraction may be resistant to atmospheric oxidation	

Soil Adsorption Coefficient (KOCWIN v2.00):

Koc :	254.7 L/kg (MCI method)
Log Koc:	2.406 (MCI method)
Koc :	107.5 L/kg (Kow method)
Log Koc:	2.032 (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: 4.33E-006 atm-m3/mole (estimated by Bond SAR Method)

Half-Life from Model River:	159 hours (6.625 days)
Half-Life from Model Lake:	1832 hours (76.35 days)

Removal In Wastewater Treatment:

Total removal:	2.16 percent
Total biodegradation:	0.09 percent
Total sludge adsorption:	1.83 percent

Total to Air:	0.24 percent

(using 10000 hr Bio P,A,S) **Level III Fugacity Model:**

	Mass Amount (percent)	Half-Life (hr)	Emissions (kg/hr)
Air	1.81	88.5	1000
Water	20.2	900	1000
Soil	77.7	1.8e+003	1000
Sediment	0.295	8.1e+003	0

Persistence Time: 1.02e+003 hr

The Ecological Categorization Results from the Canadian Domestic Substances List state that 2,3,5,6-tetramethyl pyrazine is not persistent in the environment.

Media of concern leading to Categorization	Water
Experimental Biodegradation half-life (days)	Not Available
Predicted Ultimate degradation half-life (days)	37.5
MITI probability of biodegradation	0.4558
EPI Predicted Ozone reaction half-life (days)	999
EPI Predicted Atmospheric Oxidation half-life (days)	3.689

Data accessed December 2016 on the OECD website

10.2. Aquatic toxicity

May cause long-term adverse effects in the aquatic environment.

The Ecological Categorization Results from the Canadian Domestic Substances List state that there is no inherent toxicity to aquatic organisms.

Pivotal value for iT (mg/l)	76.203
Toxicity to fish (LC50 in mg/l) as predicted by Ecosar v0.99g	76.203
Toxicity to fish (LC50 in mg/l) as predicted by Oasis Forecast M v1.10	128.9339
Toxicity to fish (LC50 in mg/l) as predicted by Aster	249.040703
Toxicity to fish (LC50 in mg/l) as predicted by PNN	354.31804
Toxicity to fish, daphnia, algae or mysid shrimp (EC50 or LC50 in mg/l) as predicted by Ecosar v0.99g	20.03
Chronic toxicity to daphnia or algae (EC50 in mg/l) as predicted by Ecosar v0.99g	4.473
Toxicity to fish (LC50 in mg/l) as predicted by Neutral Organics QSAR in Ecosar v0.99g	7.62E+001

Data accessed December 2016 on the OECD website

ECOSAR version 1.11 reports the following aquatic toxicity data for CAS RN 1124-11-4:

Values used to Generate ECOSAR Profile: Log Kow: 2.129 (EPISuite Kowwin v1.68 Estimate) Wat Sol: 4245 (mg/L, EPISuite WSKowwin v1.43 Estimate)

ECOSAR v1.11 Class-specific Estimations:

Neutral Organics

ECOSAR Class	Organism	Duration	End Pt	Predicted mg/L (ppm)
Neutral Organics :	Fish	96-hr	LC50	85.616
Neutral Organics :	Daphnid	48-hr	LC50	49.198
Neutral Organics :	Green Algae	96-hr	EC50	38.504
Neutral Organics :	Fish		ChV	8.487
Neutral Organics :	Daphnid		ChV	4.960
Neutral Organics :	Green Algae		ChV	10.355
Neutral Organics :	Fish (SW)	96-hr	LC50	107.868
Neutral Organics :	Mysid	96-hr	LC50	73.439
Neutral Organics :	Fish (SW)		ChV	12.671
Neutral Organics :	Mysid (SW)		ChV	6.109

10.3. Sediment toxicity

No data available to us at this time.

10.4. Terrestrial toxicity

ECOSAR version 1.11 reports the following terrestrial toxicity data for CAS RN 1124-11-4:

Values used to Generate ECOSAR Profile:

Log Kow: 2.129 (EPISuite Kowwin v1.68 Estimate) Wat Sol: 4245 (mg/L, EPISuite WSKowwin v1.43 Estimate)

ECOSAR v1.11 Class-specific Estimations:

Neutral Organics

ECOSAR Class	Organism	Duration	End Pt	Predicted mg/L (ppm)
Neutral Organics :	Earthworm	14-day	LC50	229.590

10.5. All other relevant types of ecotoxicity

EPISuite provides the following data:

Bioaccumulation Estimates (BCFBAF v3.01):

Log BCF from regression-based method:	0.512 (BCF = 3.247 L/kg wet-wt)
Log Biotransformation Half-life (HL):	-1.1293 days (HL = 0.07424 days)
Log BCF Arnot-Gobas method (upper trophic):	0.371 (BCF = 2.348)
Log BAF Arnot-Gobas method (upper trophic):	0.371 (BAF = 2.348)
log Kow used:	1.28 (expkow database)

The Ecological Categorization Results from the Canadian Domestic Substances List state that 2,3,5,6-tetramethyl pyrazine is not bioaccumulative in the environment.

Empirical Log Kow	1.28
Log Kow predicted by KowWin	2.13
Log BAF T2MTL predicted by Gobas	0.330460796872068
Log BCF 5% T2LTL predicted by Gobas	0.28346488346288
Log BCF Max predicted by OASIS	1.76725940723549
Log BCF predicted by BCFWIN	0.286

Data accessed December 2016 on the OECD website

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Review

The FEMA GRAS assessment of pyrazine derivatives used as flavor ingredients

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Summary

This is the fifth in a series of safety evaluations performed by the Expert Panel of the Flavor and Extract Manufacturers Association (FEMA). In 1993, the Panel initiated a comprehensive program to re-evaluate the safety of more than 1700 GRAS flavoring substances under conditions of intended use. Elements that are fundamental to the safety evaluation of flavor ingredients include exposure, structural analogy, metabolism, pharmacokinetics and toxicology. Flavor ingredients are evaluated individually taking into account the available scientific information on the group of structurally related substances. Scientific data relevant to the safety evaluation of the use of pyrazine derivatives as flavoring ingredients is evaluated. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Pyrazine derivatives; Flavoring agents; Food safety; GRAS

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Abbreviations: AO, aldehyde oxidase; CHO, Chinese hamster ovary; CYP-450, cytochrome P450; DNA, deoxyribonucleic acid; F, female; FEMA, The Flavor and Extract Manufacturers Association; FMO, flavin-containing monooxygenases; GRAS, generally recognized as safe; GRASa, GRAS affirmed; GRASr, GRAS reaffirmed; M, male; NAS, National Academy of Sciences; NCI, National Cancer Institute; NOAEL, no-observed-adverse-effect level; NOEL, no-

observed-effect level; NR, not reported; LD₅₀, median lethal dose; MLA, mouse lymphoma cells; ppm, parts per million; PCV, pack cell volume; SAL, *Salmonella typhimurium*; SCE, sister chromatid exchanges; SLR, scientific literature review; XO, xanthine oxidase.

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1. Safety evaluation of pyrazine derivatives used as flavoring ingredients

1.1. Chemical identity

This summary presents the key data relevant to the safety evaluation of 41 pyrazine derivatives (Table 1). Pyrazines are monocyclic heteroaromatic substances containing nitrogen atoms in the 1- and 4-position of the aromatic ring. All substances in this group possess a pyrazine or quinoxaline (benzene ring fused to a pyrazine ring) ring. Forty of the 41 pyrazine derivatives are ring substituted with one or more alkyl, alicyclic, acetyl, alkoxy and/or alkyl thiol/sulfide ring substituents. The remaining substance pyrazine (No. 40) is unsubstituted. Based on available chemical, metabolic and toxicological data, the group of pyrazine derivatives has been organized into four structural subcategories:

- unsubstituted pyrazine (No. 40);
- pyrazine derivatives containing a hydrocarbon (alkyl, alicyclic or alkylaryl) substituent (Nos 1–22 and Nos 33, 39 and 41);
- pyrazine derivatives containing an oxygenated functional group and aliphatic side chain (Nos 23–32, 34);
- pyrazine derivatives containing a thiol or sulfide functional group in the aliphatic side chain (Nos 35–38).

Pyrazine derivatives participate in common pathways of metabolic detoxication principally involving oxidation of side-chain alkyl or oxygenated functional groups and hydroxylation of the ring (see Section 1.3.2). Results of acute, subchronic, and chronic toxicity studies are consistent with known biochemical fate of these substances in animals.

1.2. Exposure

1.2.1. Flavor use

Pyrazines are important contributors to the flavor of various roasted, toasted, or similarly heated foods. They are a common constituent of foods, and are thought to arise primarily from a heat-induced condensation between amino acids and sugars (α -dicarbonyl com-

pounds), through the Strecker degradation (Fisher and Scott, 1997). Their concentrations in foods are in the range from approximately 0.001 to 40 ppm (CIVO–TNO, 1999). Pyrazine derivatives exhibit a wide variety of aromas in food. For instance, 2-methoxy-3-isopropylpyrazine produces a green pea odor, 2-acetylpyrazine contributes a popcorn-like odor, and dimethyl- and trimethylpyrazines exhibit the roast aroma of nuts and coffee, respectively (Bauer and Garbe, 1985). Aroma thresholds (i.e. the lowest concentration at which a flavor of the detected compound is recognized) for pyrazine derivatives vary in concentration by as many as eight orders of magnitude. Aroma thresholds in water are in the range from 1×10^{-3} ppb for 2-methoxy-3-hexylpyrazine to 1.8 ppm for 2,5-dimethylpyrazine (No. 6) to 175 ppm for pyrazine itself (No. 40) (Seifert et al., 1970).

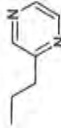
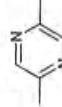
The total annual volume of use of pyrazine and quinoxaline derivatives as flavoring ingredients is 2135 kg in the USA (Lucas et al., 1999). Approximately two-thirds of the total annual volume arises from use of three substances (2,3,5-trimethylpyrazine (No. 14), 347 kg; 2,3,5,6-tetramethylpyrazine (No. 20), 144 kg; and acetylpyrazine (No. 24), 923 kg). Production volumes and intake levels of individual flavoring substances are reported in Table 1. None of the substances has an estimated daily per capita intake¹ (“eaters only”) of greater than 3 $\mu\text{g}/\text{kg}$ body weight per day from use as a flavoring agent.

1.2.2. Natural occurrence

Thirty-four of the substances in this group have been reported to occur naturally in foods (CIVO–TNO, 1999). They have been detected in asparagus, potato, kohlrabi and wheat bread (CIVO–TNO, 1999). Quantitative

¹ Intake ($\mu\text{g}/\text{kg}$) calculated as follows: (((annual volume, kg) $\times$ (1×10^9 $\mu\text{g}/\text{kg}$)) / (population $\times$ survey correction factor $\times$ 365 days)), where population (10%, “eaters only”) = 26×10^6 for the USA. The survey correction factor of 0.8 represents the assumption that 80% of the flavor volume was reported in the survey (Lucas et al., 1999). For substances that were not surveyed the anticipated volume was used. Intake ($\mu\text{g}/\text{kg}$ body weight per day) calculated as follows: (($\mu\text{g}/\text{day}$)/body weight), where body weight = 60 kg. Slight variations may occur from rounding off.

Table 1
Identity and exposure data for pyrazine derivatives

Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume (kg)	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods (kg)	Consumption ratio
				µg/day	µg/kg bw/day		
1. 2-Methylpyrazine	3309	109-08-0 	50	7	0.1	114,486	2290
2. 2-Ethylpyrazine	3281	13925-00-3 	44	6	0.1	21,690	493
3. 2-Propylpyrazine	3961	18138-03-9 	1	0.1	0.002	+	NA
4. 2-Isopropylpyrazine	3940	29460-90-0 	1	0.1	0.002	+	NA
5. 2,3-Dimethylpyrazine	3271	5910-89-4 	27	4	0.1	7679	284
6. 2,5-Dimethylpyrazine	3272	123-32-0 	59	8	0.1	37,078	628
7. 2,6-Dimethylpyrazine	3273	108-50-9 	18	2	0.04	47,562	2642
8. 2-Ethyl-3-methylpyrazine	3155	15707-23-0 	72	9	0.2	18,074	251

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Table 1 (continued)

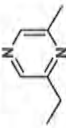
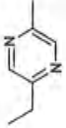
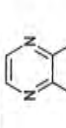
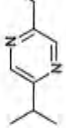
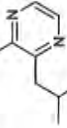
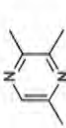
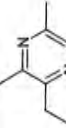
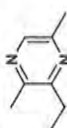
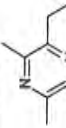
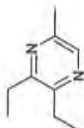
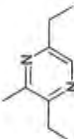
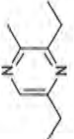
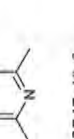
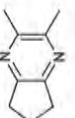
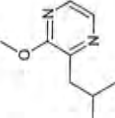
Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume (kg)	Daily per capita intake ("eaters only") µg/day	Daily per capita intake ("eaters only") µg/kg bw/day	Annual volume in naturally occurring foods (kg)	Consumption ratio
9. 2-Ethyl-6-methylpyrazine	3919	13925-03-6 	3	0.4	0.01	+	NA
10. 2-Ethyl-5-methylpyrazine	3154	13360-64-0 	6	1	0.01	4,793	799
11. 2,3-Diethylpyrazine	3136	15707-24-1 	5	1	0.01	+	NA
12. 2-Methyl-5-isopropylpyrazine	3554	13925-05-8 	3	0.4	0.01	+	NA
13. 2-Isobutyl-3-methylpyrazine	3133	13925-06-9 	0.05	0.01	0.0001	166	3320
14. 2,3,5-Trimethylpyrazine	3244	14667-55-1 	347	46	0.8	22,650	65
15. 2-Ethyl-(3, 5 or 6)-dimethylpyrazine	3149	13925-07-0  13360-65-1 	72	9	0.2	7082	98
16. 3-Ethyl-2,6-dimethylpyrazine	3150	13925-07-0 	2	0.3	0.004	9655	4828

Table 1 (continued)

Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume (kg)	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods (kg)	Consumption ratio
				µg/day	µg/kg bw/day		
17. 2,3-Diethyl-5-methylpyrazine	3336	18138-04-0 	5	1	0.001	+	NA
18. 2,5-Diethyl-3-methylpyrazine	3915	32736-91-7 	0.1	0.01	0.0002	+	NA
19. 3,5-Diethyl-2-methylpyrazine	3916	18138-05-1 	0.1	0.01	0.0002	+	NA
20. 2,3,5,6-Tetramethylpyrazine	3237	1124-11-4 	144	19	0.3	7740	54
21. 5-Methyl-6,7-dihydro-5H-cyclopentapyrazine	3306	32747-48-0 	34	4	0.1	+	NA
22. 6,7-Dihydro-2,3-dimethyl-5H-cyclopentapyrazine	3917	38917-63-4 	0.1	0.01	0.0002	+	NR
23. 2-Isobutyl-3-methoxypyrazine	3132	24683-00-9 	7	1	0.02	110	16

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Table 1 (continued)

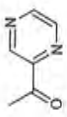
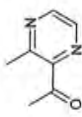
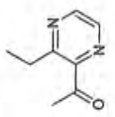
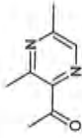
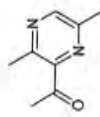
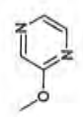
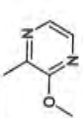
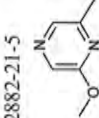
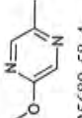
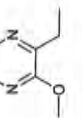
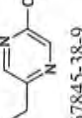
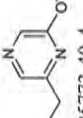
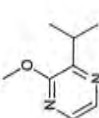
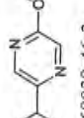
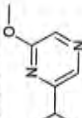
Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume (kg)	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods (kg)	Consumption ratio
				µg/day	µg/kg bw/day		
24. Acetylpyrazine	3126	22047-25-2 	923	122	2	1882	2
25. 2-Acetyl-3-methylpyrazine		23787-80-6 	0.5	0.1	0.001	+	NA
26. 2-Acetyl-3-ethylpyrazine	3250	32974-92-8 	1	0.1	0.002	+	NA
27. 2-Acetyl-(3, 5 or 6)-dimethylpyrazine	3327	54300-09-3  54300-08-2 	4	1	0.01	+	NA
28. Methoxypyrazine	3302	3149-28-8 	5	1	0.01	+	NA

Table 1 (continued)

Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume (kg)	Daily per capita intake ("eaters only") µg/day	µg/kg bw/day	Annual volume in naturally occurring foods (kg)	Consumption ratio
29. (2 or 5 or 6)-Methoxy-3-methylpyrazine	3183	2847-30-5 	113	15	0.2	+	NA
		2882-21-5 					
		2882-22-6 					
30. 2-Ethyl-(3 or 5 or 6)-methoxypyrazine	3280	25680-58-4 	11	1	0.02	+	NA
		68039-50-9 					
		67845-38-9 					
31. 2-Methoxy-(3 or 5 or 6)-isopropylpyrazine	3358	25773-40-4 	0.5	0.01	0.001	1	2
		56891-99-7 					
		68039-46-3 					

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Table 1 (continued)

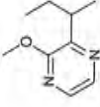
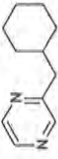
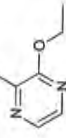
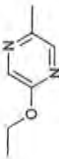
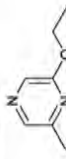
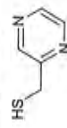
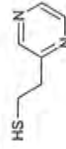
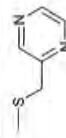
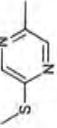
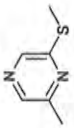
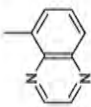
Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume (kg)	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods (kg)	Consumption ratio
				µg/day	µg/kg bw/day		
32. 2-Methoxy-3-(1-methylpropyl)-pyrazine	3433	24168-70-5 	1	0.1	0.002	+	NA
33. (Cyclohexylmethyl) pyrazine	3631	28217-92-7 	0.1	0.01	0.0002	—	NA
34. 2-Methyl-(3 or 5 or 6)-ethoxypyrazine	3569	32737-14-7 	0.05	0.01	0.0001	—	NA
		67845-34-5 					
		53163-97-6 					
35. 2-(Mercaptomethyl) pyrazine	3299	59021-02-2 	0.05	0.01	0.0001	—	NA
36. 2-Pyrazinylethane thiol	3230	35250-53-4 	6	1	0.01	—	NA
37. Pyrazinylmethyl methyl sulfide	3231	21948-70-9 	0.05	0.01	0.0001	—	NA

Table 1 (continued)

Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume (kg)	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods (kg)	Consumption ratio
				µg/day	µg/kg bw/day		
38. (3 or 5 or 6)-(Methylthio)-2-methylpyrazine	3208	2882-20-4 	99	13	0.2	—	NA
		2884-14-2 					
		2884-13-1 					
39. 5-Methylquinoxaline	3203	13708-12-8 	5	1	0.01	140	28
40. Pyrazine		290-37-9 	1.2	0.2	0.003	42,600	35,000
41. 5,6,7,8-Tetrahydro-quinoxaline	3321	34413-35-9 	64	8	0.1	—	NA

natural occurrence data has been reported for 17 of the substances (see Table 1), and indicate that intake of those substances is predominantly from food (i.e. consumption ratio greater than 1). Consumption of the parent substance pyrazine (No. 40) from food is greater than 35,000 times its intake as a flavoring substance (Stofberg and Kirschman, 1985; Stofberg and Grund-schober, 1987).

1.3. Absorption, distribution, metabolism and excretion

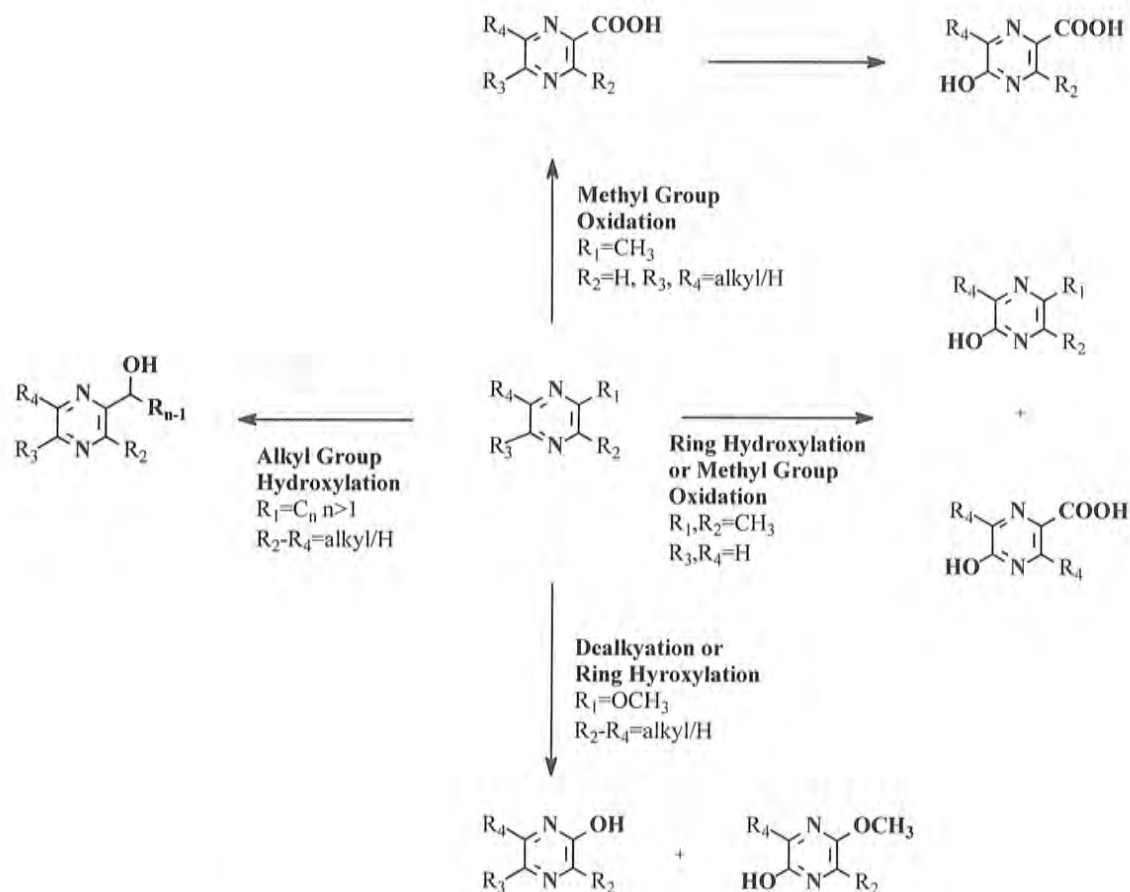
1.3.1. Absorption, distribution and excretion

Pyrazine is a weaker base ($pK_b = 13.4$) than pyridine ($pK_b = 8.8$), pyrimidine ($pK_b = 12.7$) or pyridazine ($pK_b = 11.7$) (Damani and Crooks, 1982). At intestinal pH (5–7), absorption of weak amine bases such as pyrazine derivatives is optimal (Schranker et al., 1957; Hogben et al., 1959). In humans and laboratory rodents, orally administered substituted pyrazines are rapidly absorbed from the gastrointestinal tract and excreted (Hawksworth and Scheline, 1975; Sjödin et al., 1989). Approximately 90% of the dose (100 mg/kg) of

2-methylpyrazine (No. 1), 2,5-dimethylpyrazine (No. 6), 2,6-dimethylpyrazine (No. 7) or methoxypyrazine (No. 28) administered to male Wistar rats by stomach tube was excreted in the urine as polar metabolites within 24 h. Greater than 50% of the administered dose (100 mg/kg) of 2,3-dimethylpyrazine (No. 5) was recovered in the urine within 24 h (Hawksworth and Scheline, 1975). Data available on larger, fused pyrazine derivatives also indicate that these materials are absorbed, distributed and excreted rapidly and efficiently following oral administration to the rat (Sjödin et al., 1989) and human (Renberg et al., 1989).

1.3.2. Metabolism

1.3.2.1. Alkyl, alicyclic and alkylaryl substituted pyrazine derivatives (Nos 1–22, 33, 39). The biotransformation of the above-referenced substituted pyrazines is expected to occur primarily via oxidation of the side-chain (see Fig. 1). An alternative pathway for substituted pyrazines and primary pathway for pyrazine (No. 40) itself involves hydroxylation of the pyrazine ring (Hawksworth and Scheline, 1975; Whitehouse et



*Excretion products in bold

Fig. 1. Metabolism of alkyl- and alkoxy-substituted pyrazine derivatives.*

al., 1987; Yamamoto et al., 1987a,b). *N*-Oxygenation of pyrazines by cytochrome P-450 (CYP-450) has not been observed (Hawthornth and Scheline, 1975). Detoxication of alkyl-substituted pyrazines via side-chain oxidation and ring hydroxylation is comparable to the metabolic detoxication of alkyl-substituted pyridines in animals (Hawthornth and Scheline, 1975; Caputo et al., 1988, 1989; Blake and Beattie, 1989a; Renberg et al., 1989; Oldham et al., 1990; Weidolf et al., 1992).

Methyl-substituted pyrazines are oxidized to yield the corresponding pyrazine-2-carboxylic acids. At least 89% of a 100 mg/kg oral dose of 2-methylpyrazine (No. 1), 2,5-dimethylpyrazine (No. 6) or 2,6-dimethylpyrazine (No. 7) was metabolized in the rat by side-chain oxidation to yield the corresponding pyrazine-2-carboxylic acid derivative. The acids were mainly excreted unconjugated, although 10–15% of the administered dose of 2-methylpyrazine and 2,5-dimethylpyrazine were excreted as the corresponding glycine conjugates (Hawthornth and Scheline, 1975). Side-chain oxidation of methylpyrazine derivatives to yield the corresponding alcohols has been demonstrated for other pyrazine derivatives (Turesky et al., 1988; Knize et al., 1989; Sjödin et al., 1989; Wallin et al., 1989).

Alkyl-ring substituents ($>C_1$) are expected to undergo CYP-450 catalyzed oxidation mainly at the carbon directly adjacent to the pyrazine ring to yield the corresponding secondary alcohol (Caputo et al., 1988, 1989; Parkinson, 1996). The corresponding secondary alcohol may be further oxidized to the corresponding ketone. Reduction of the ketone by cytoplasmic carbonyl reductase is favored in vivo (Farrelly et al., 1987; Parkinson, 1996). Therefore, it would be anticipated that the alcohol metabolite would be excreted either unchanged or conjugated in the urine.

Alicyclic-substituted pyrazines, such as 6,7-dihydro-2,3-dimethyl-5*H*-cyclopentapyrazine (No. 22), are also expected to undergo side-chain oxidation similar to that previously described for alkyl-substituted pyrazines ($>C_1$). In addition, hydroxylation at various positions on the alicyclic ring is likely, based on reports of similar hydroxylation reactions for alicyclic substances in a variety of in vitro and in vivo test systems (Governa et al., 1987; Kirk et al., 1987; Muktar et al., 1987; Rogiers et al., 1987). Products of oxidative metabolism may be excreted unchanged or conjugated with glycine, glucuronic acid or sulfate prior to excretion (Caputo et al., 1989; Parkinson, 1996).

Alkyl-substituted pyrazines may undergo ring hydroxylation as an alternative pathway when other routes of detoxication are less favorable. For example, 2,5- and 2,6-dimethylpyrazine are oxidized almost exclusively in rats via their aliphatic side-chains to carboxylic acid derivatives. Conversely, 2,3-dimethylpyrazine primarily undergoes ring hydroxylation, because side-chain oxidation is impaired (only 13% of the administered dose

oxidized) by the steric hindrance of the methyl groups (Hawthornth and Scheline, 1975).

Ring hydroxylation is catalyzed by the molybdenum hydroxylases, xanthine oxidase (XO) and aldehyde oxidase (AO), which are present in the cytosol of humans and other mammalian species, predominantly in the liver. These enzymes catalyze ring hydroxylation of a wide range of endogenous and exogenous *N*-heterocyclics bearing a substituent and/or a second fused ring.

The molybdenum hydroxylases facilitate oxidation reactions involving nucleophilic attack by an oxygen (OH^-) derived from water. Oxidation occurs at the most electropositive atom, which, in *N*-heterocyclics, is generally the carbon adjacent to the ring nitrogen. The role of the molybdenum hydroxylases increases as the number of ring nitrogen atoms increase since each nitrogen activates the ring system towards nucleophilic attack. The oxidation action of the molybdenum hydroxylases is opposite from the microsomal monooxygenases (such as CYP-P450), which catalyze electrophilic attack by an oxygen atom derived from molecular oxygen (O_2) (Beedham, 1988). While substituted-monocyclic pyrazines may be substrates for the molybdenum hydroxylases when other pathways are unfavorable (Hawthornth and Scheline, 1975), bicyclic heteroaromatic (e.g. quinoxaline) substances are their preferred substrates (Beedham, 1988). Quinoxaline (i.e. 2,3-benzopyrazine) incubated in vitro with rabbit liver aldehyde oxidase is ring hydroxylated to yield 2-hydroxyquinoxaline and 2,3-dihydroxyquinoxaline (Stubley et al., 1979). The structurally related bicyclic 5-methylquinoxaline (No. 39) would be expected to undergo ring hydroxylation in addition to methyl group oxidation.

1.3.2.2. Pyrazine (No. 40) or oxygenated pyrazine derivatives (Nos 23–32, 34). Pyrazine or pyrazine derivatives with a ring-activating alkoxy side-chain, such as 2-methoxypyrazine, are more susceptible to nucleophilic attack by the molybdenum hydroxylases (Beedham, 1988) and, therefore, primarily undergo ring hydroxylation (see Fig. 1). Additionally, the methoxy side-chain is *O*-demethylated. In rats, approximately 75% of a 100 mg/kg body weight oral dose of 2-methoxypyrazine undergoes ring hydroxylation (Hawthornth and Scheline, 1975), while 20% is accounted for by *O*-demethylation. *O*-Demethylation of the methoxypyridine moiety has also been reported (Blake and Beattie, 1989b).

Ring hydroxylation of the antitubercular agent pyrazinamide has been reported in vitro (Yamamoto et al., 1987b) and in vivo (Whitehouse et al., 1987; Yamamoto et al., 1987a) in both humans and rats. A dose of approximately 12.5 mg pyrazinamide/kg body weight given orally to one human was hydrolyzed to pyrazine-

2-carboxylic acid (35% of dose) and ring hydroxylated to yield 5-hydroxypyrazine-2-carboxylic acid (25% of dose) (Whitehouse et al., 1987). The hydroxylation of pyrazinamide and pyrazanoic acid in vitro to form 5-hydroxypyrazinamide and 5-hydroxypyrazine-2-carboxylic acid, respectively, occurred in the presence of xanthine oxidase-rich human liver cytosol (Yamamoto et al., 1987b).

In rats, 3-acetylpyridine is mainly reduced to the secondary alcohol and excreted as the glucuronic acid conjugate (Damani et al., 1980; Schwartz et al., 1978). Therefore, the structurally-related acylated pyrazines, such as 2-acetyl-3-methylpyrazine (No. 25), are likely to metabolize by reduction of the ketone functional group. Alternatively, the terminal methyl group may be oxidized to yield the corresponding carboxylic acid.

1.3.2.3. Pyrazines with ring substituents containing a thiol or sulfide functional group (Nos 35–38). The presence of sulfur in the side-chain of pyrazines and alkylpyrazines provides a further metabolic option. The reactive lone pair of electrons on divalent sulfur in thiols and monosulfides permits rapid oxidation. Alkyl and aromatic sulfides are oxidized to sulfoxides and then to sulfones (Hoodi and Damani, 1984; Nickson and Mitchell, 1994; Nickson et al., 1995). The oxidation to sulfoxides is catalyzed by at least three enzyme systems, CYP-450, microsomal prostaglandin synthetase, and the flavin-containing monooxygenases (FMO) (Ziegler, 1980; Cashman and Williams, 1990; Cashman et al., 1990, 1995a,b; Rettie et al., 1990; Yoshihara and Tatsumi, 1990; Sadeque et al., 1992, 1995; Nickson and Mitchell, 1994; Elfarrar et al., 1995; Nnane and Damani, 1995). However, for simple aliphatic, alicyclic and aromatic sulfides, oxidation is primarily catalyzed by FMO and, to a lesser extent, by CYP-450 (Hoodi and Damani, 1984). Subsequent oxidation of the sulfoxide to the sulfone is an irreversible reaction (Williams et al., 1966; Damani, 1987). Essentially, all low molecular weight aliphatic and aromatic sulfones are metabolically stable. Hence, sulfoxides and sulfones are excreted in the urine of animals exposed to sulfides. Thiols (Nos 35 and 36) are very reactive substances. In vivo, they become even more reactive mainly because most thiols exist in the ionized form at physiologic pH. Metabolic options for thiols include: oxidation to form unstable sulfenic acids (RSOH) which may be oxidized to sulfinic acid (RSO₂H) and sulfonic acid (RSO₃H); methylation to yield methyl sulfides which then form sulfoxides and sulfones; reaction with physiologic thiols to form mixed disulfides and conjugation with glucuronic acid; or oxidation of the α -carbon, which results in desulfuration and the formation of an aldehyde (McBain and Menn, 1969; Dutton and Illing, 1972; Maiorino et al., 1988; Richardson et al., 1991).

1.4. Toxicological studies

1.4.1. Acute toxicity

Acute oral rat LD₅₀ values are available for 17 of the 41 pyrazines discussed in this review (Table 2). The rat acute oral LD₅₀ values indicate a low level of toxicity for substituted pyrazines, ranging from 158 mg/kg for thiol derivative 2-pyrazinylethanethiol (No. 36) to greater than 4000 mg/kg for 2-isobutyl-3-methoxypyrazine (No. 23); however, almost all of the LD₅₀ values are in the narrower range of approximately 500 to 2500 mg/kg (Wheldon et al., 1967; Oser, 1969e; Roure Inc., 1974; Posternak et al., 1975; Moran et al., 1980; Burdock and Ford, 1990).

All the available mouse acute oral LD₅₀ values are 2000 mg/kg or greater and confirm the low toxicity of these pyrazines in a second species (Babish, 1978a; Quest International, 1983a,b).

1.4.2. Short-term toxicity

Ninety-day or 13-week dietary studies are available for 17 of the 41 pyrazines discussed in this review (Table 2). Fifteen of the 17 studies were performed at a single target intake level that was 100 times the estimated possible average daily intake (PADI) from use of the substance as a flavoring substance. The PADI is determined by (1) multiplying usual use levels of the substance in each of 33 food categories (e.g. baked goods and meat products) times the average amount of that food category consumed daily and (2) summing the intake over all 33 food categories (United States Department of Agriculture, 1965). For the vast majority of flavoring substances that have low reported annual volumes of use (IOFI, 1995; Lucas et al., 1999), the PADI is a gross exaggeration of the average daily intake. The PADI calculation assumes that all foods in a food category always contain that flavoring substance and that the food category is consumed each day (Oser and Hall, 1977). Therefore, the feeding level in these studies is many orders of magnitude greater than actual intake levels of pyrazine derivatives as flavoring substances.

The subchronic studies are available for a structurally diverse group of substituted pyrazine derivatives. Subcategories of pyrazine derivatives studied include:

1. 11 studies used alkyl-, alkylaryl- or alicyclic-substituted pyrazines (Wheldon et al., 1967; Oser, 1969a,b,c,d; 1970; Posternak et al., 1969, 1975; Babish, 1978b);
2. three studies used methoxy- or acetyl-substituted pyrazines (Posternak et al., 1969, 1975; Osborne et al., 1981);
3. three studies used thiol or sulfide-substituted alkylpyrazines (Posternak et al., 1975).

Table 2
Acute and short-term toxicity studies for pyrazine derivatives

Substance	Oral acute studies Oral LD ₅₀ mg/kg bw (species)	Reference	Short-term studies Species, sex ^a	Time (days)/route	NOEL (mg/kg bw)	Reference
1. 2-Methylpyrazine	1800 (Rat)	Moran et al. (1980)				
5. 2,3-Dimethylpyrazine	613 (Rat)	Moran et al. (1980)				
6. 2,5-Dimethylpyrazine	1020 (Rat)	Moran et al. (1980)				
7. 2,6-Dimethylpyrazine	880 (Rat)	Moran et al. (1980)				
8. 2-Ethyl-3-methylpyrazine	600 (Rat)	Moran et al. (1980)	Rat, M, F	90/diet	5.31 (M) 5.22 (F)	Posternak et al. (1969)
10. 2-Ethyl-5-methylpyrazine	900 (Rat)	Moran et al. (1980)	Rat, M, F	90/diet	18 (M) ND ^b (F)	Oser (1969a)
11. 2,3-Diethylpyrazine			Rat, M, F	90/diet	1.75	Posternak et al. (1969)
14. 2,3,5-Trimethylpyrazine	806 (Rat)	Moran et al. (1980)	Rat, M, F	90/diet	18	Oser (1969b)
15. 2-Ethyl-(3, 5 or 6)-dimethylpyrazine	456 (Rat)	Moran et al. (1980)	Rat, M, F	90/diet	18	Oser (1969c)
16. 3-Ethyl-2,6-dimethylpyrazine	504 (Rat)	Posternak et al. (1975)	Rat, M, F	84/diet	12.7 (M) 12.3 (F)	Posternak et al. (1975)
20. 2,3,5,6-Tetramethylpyrazine	1910 (Rat)	Oser (1969e)	Rat, M, F	90/diet	55 (M) ND ^c (F)	Oser (1969d)
21. 5 <i>H</i> -5-Methyl-6,7-dihydrocyclopentapyrazine	820 (Rat)	Wheldon et al. (1967)	Rat, M	90/diet	50	Wheldon et al. (1967)
23. (Cyclohexylmethyl)pyrazine	2673 (Mouse)	Oser (1978a)	Rat, M, F	90/diet	0.44 (M) 0.47 (F)	Posternak et al. (1975)
24. Acetylpyrazine	> 3000 (Rat)	Posternak et al. (1975)	Rat, M, F	91/diet	8.2 ^d	Osborne, 1981
28. Methoxypyrazine			Rat	91/diet	20	Posternak et al. (1969)
29. (2, 5 or 6)-Methoxy-3-methylpyrazine			Rat, M, F	90/diet	45 (M) 53 (F)	
32. 2-Methoxy-3-(1-methylpropyl)pyrazine	2000 (Mouse)	Quest International (1983b)				
33. 2-Isobutyl-3-methoxypyrazine	> 4000 (Rat)	Roure, Inc. (1974)				
	2000 (Mouse)	Quest International (1983b)				
35. 2-(Mercaptomethyl)pyrazine	2100 (Rat)	Burdock and Ford (1990)				
36. 2-Pyrazinylethanethiol	158 (Rat)	Posternak et al. (1975)	Rat, M, F	91/diet	(M) 16.30 (F)	Posternak et al. (1975)
37. Pyrazinylmethyl methyl sulfide	2500 (Rat)	Posternak et al. (1975)	Rat, M, F	91/diet	1.66 (M) 1.63 (F)	Posternak et al. (1975)
38. (3, 5 or 6)-(Methylthio)-2-methylpyrazine	1970 (Rat)	Posternak et al. (1975)	Rat, M, F	91/diet	4	Posternak et al. (1975)
39. 5-Methylquinoxaline			Rat, M, F	90/diet	17.1	Posternak et al. (1969)

^a M, male; F, female. If not listed, sex was not specified in the report.

^b NOEL not determined in females; decrease in food utilization efficiency observed at 18 mg/kg/day.

^c NOEL not determined in females; growth rate and food utilization efficiency effects observed at 55 mg/kg/day.

^d This study was performed using a single dose level. Therefore, this dose level is not a true NOEL, but is the highest dose tested that produced no effects. The actual NOEL would be higher.

There was no evidence of histopathologic changes in any of the single-dose short-term studies. The only consistent effects observed among six of these studies were slight to moderate decreases in growth rates and efficiency of food utilization due to palatability problems.

1.4.2.1. Alkyl-, Alkylaryl- and alicyclic-substituted pyrazines and alkyl-substituted pyrazines

1.4.2.1.1. 2-Ethyl-5-methylpyrazine (No. 10), 2,3,5-trimethylpyrazine (No. 14), 2-ethyl-(3,5 or 6)-dimethylpyrazine (No. 15) and 2,3,5,6-tetramethylpyrazine (No. 20). These di-, tri- and tetra-alkyl substituted pyrazines were studied under a similar protocol in rat dietary 90-day studies (Oser, 1969a,b,c,d). A control and a test group, each consisting of 15 male and 15 female albino weanling rats (Food and Drug Research Labs strain), were maintained individually in temperature and humidity controlled housing with ad lib. access to water and food. The concentration of the test material in the diet was adjusted every 2 weeks to maintain a constant level of dietary intake of approximately 15 mg/kg body weight per day for 2-ethyl-5-methylpyrazine (No. 10), 2,3,5-trimethylpyrazine (No. 14) and 2-ethyl-3,(5 or 6)-dimethylpyrazine (No. 15) or 44 mg/kg body weight per day for 2,3,5,6-tetramethylpyrazine (No. 20). Clinical observations were recorded daily and food consumption and body weights were determined weekly. During weeks 6 and 12 of the study, hematological examinations, clinical chemistry determinations and urine analyses were performed on 10 animals of each sex. After 90 days, all animals were killed and subjected to detailed necropsy examination. Tissues and organs from each animal were preserved and histopathological examinations were performed on major organs and tissues.

The only findings for female rats fed 2-ethyl-5-methylpyrazine or 2,3,5,6-tetramethylpyrazine at actual dietary doses of 18 or 55 mg/kg body weight per day, respectively, were decreased growth rates and efficiency of food utilization. These changes were not accompanied by any evidence of pathology. No effects were reported for male rats fed these substances at similar dose levels (17 and 50 mg/kg body weight per day, respectively) for 90 days. Dietary intake of 18 mg 2,3,5-trimethylpyrazine or 2-ethyl-(3, 5 or 6)-dimethylpyrazine/kg body weight per day for 90 days resulted in no differences between test and control groups for either sex. The actual intake level of 17–18 or 50–55 mg/kg body weight per day is at least 1000 times the daily per capita intake¹ (“eaters only”) of each of the 4 alkyl-substituted pyrazines from use as flavoring substances (Table 2).

1.4.2.1.2. 2-Ethyl-3-methylpyrazine (No. 8), 2,3-diethylpyrazine (No. 11), 3-ethyl-2,6-dimethylpyrazine (No. 16) and 5-methylquinoxaline (No. 39). Three alkyl-substituted pyrazines were studied under a similar protocol

in rat dietary 90-day studies (Posternak et al., 1969, 1975). A control and a test group, each consisting of 16 male and female Charles River CD rats, were housed in pairs of the same sex and given ad lib. access to water and food. The concentration of the test material in the diet was adjusted during the study to maintain constant levels of dietary intake of 5.31 male (M) and 5.22 female (F) mg/kg/day for 2-ethyl-3-methylpyrazine (Posternak et al., 1969), 1.75 mg/kg body weight per day for 2,3-diethylpyrazine (Posternak et al., 1969), 12.7 (M) and 12.3 (F) mg/kg body weight per day for 3-ethyl-2,6-dimethylpyrazine (Posternak et al., 1975) and 17.1 mg/kg body weight per day for 5-methylquinoxaline (Posternak et al., 1969). The doses were calculated to be greater than 100 times the PADIs from use as flavoring substances. Clinical observations were recorded daily and food consumption and body weights were determined weekly. During weeks 7 and 13 of the study, hematological examination and clinical chemistry (blood urea) parameters were measured. After 90 days, all animals were killed, subjected to a detailed necropsy examination and liver and kidney weights were measured. A wide range of tissues and organs from each animal were preserved and histopathological examinations were performed on major organs and tissues.

Based on growth, food intake, hematological and clinical chemistry parameters, and organ weights or organ pathology, no differences were observed between groups of control animals and those treated with 2-ethyl-3-methylpyrazine, 2,3-diethylpyrazine, or 5-methylquinoxaline. Males and females maintained on diets providing approximately 12.5 mg 3-ethyl-2,6-dimethylpyrazine/kg body weight per day exhibited decreased growth rates and a moderate reduction in efficiency of food utilization. However, hematology, clinical chemistry, organ weights and histopathology were unremarkable compared with those of the control group. Reduced body weights in test animals were not accompanied by any evidence of toxicity. The authors concluded that the reduced body weight gains were not of toxicological significance and were the result of poor palatability. The intake level 5.31 (M) and 5.22 (F) mg of 2-ethyl-3-methylpyrazine/kg body weight per day, 1.75 mg 2,3-diethylpyrazine/kg body weight per day, 12.7 (M) and 12.3 (F) mg 3-ethyl-2,6-dimethylpyrazine/kg body weight per day, or 17.1 mg 5-methylquinoxaline/kg body weight per day is at least 1000 times the daily per capita intake¹ (“eaters only”) from use of each of the three pyrazine derivatives as a flavoring substance.

1.4.2.1.3. (Cyclohexylmethyl)pyrazine (No. 33) 5,6,7,8-tetrahydroquinoxaline (No. 41) and 5-methyl-6,7-dihydro-5H-cyclopentapyrazine (No. 21). Ninety-day studies have been performed for (cyclohexylmethyl)pyrazine (No. 33), 5-methyl-6,7-dihydro-5H-cyclopentapyrazine (No. 21) and 5,6,7,8-tetrahydroquinoxaline (No. 41).

(Cyclohexylmethyl)pyrazine (Babish, 1978b) and 5,6,7,8-tetrahydroquinoxaline (Oser, 1970) were studied according to the previously described rat dietary 90-day toxicity protocol (Oser, 1969a,b,c,d). However, in the (cyclohexylmethyl)pyrazine study, the Sprague–Dawley Blu strain of albino weanling rats were used as the test species and additional organ weights (i.e. spleen and adrenal glands) were measured. (Cyclohexylmethyl)pyrazine was added to the diet at levels calculated to provide an average daily intake of 0.44 and 0.47 mg/kg body weight for the males and females, respectively. A transient significant increase in blood urea nitrogen was measured for female animals during week 6 of the study; however, the level was within the historical control range for the laboratory. Compared with control groups, an increase in relative (percent of body weight) kidney and liver weights were observed in treated males, but not in treated females. No treatment-related microscopic effects were seen for these organs or any of the other tissues examined. Based on these results, the authors of the study concluded that the intake of 0.44 or 0.47 mg (cyclohexylmethyl)pyrazine/kg body weight per day resulted in no adverse effects to male and female rats, respectively.

In a 90-day study, 5,6,7,8-tetrahydroquinoxaline (No. 41) (Oser, 1970) was added to the diet of rats at levels calculated to result in an average daily intake of 18.6 and 19.3 mg/kg body weight per day for males and females, respectively. Measurements of growth rate and food intake, hematological examinations, clinical chemistry determinations, measurement of liver and kidney weights and gross and histopathological examinations failed to reveal any significant differences between test and control animals. The intake levels of 18.6 and 19.3 mg/kg body weight per day for males and females, respectively, that resulted in no effects are at least 100,000 times the daily per capita intake¹ (“eaters only”) of 1.4×10^{-4} mg/kg body weight per day from use of 5,6,7,8-tetrahydroquinoxaline as a flavoring substance.

In a 13-week feeding study with 5-methyl-6,7-dihydro-5H-cyclopentapyrazine (No. 21), control and test groups consisted of 10 male Charles River CD rats. The rats were housed five to a cage and given ad lib. access to water and food. The target concentrations of the 5-methyl-6,7-dihydro-5H-cyclopentapyrazine in the rat diet were 100 times the maximum estimated daily human dietary intake for the low dose; 1000 times the maximum estimated daily human dietary intake for the mid-dose; and 0.5 times of the oral LD₅₀ for the high dose. Based on these, target diets were prepared to contain 100 ppm (approx. 5 mg/kg body weight per day), 1000 ppm (approx. 50 mg/kg body weight per day) and 8200 ppm (approx. 410 mg/kg body weight per day) (Wheldon et al., 1967).

Daily observations of appearance, behavior, appetite, gross signs of toxic effects and mortality were similar

among test and control animals. Weekly measurement of body weights and food consumption revealed transient reduction in food consumption in the high-dose group during the first 3 weeks that was attributed to the inappetence of the diet. Body weight gain for the high-dose animals, in particular, was reduced compared with the control animals but efficiency of food utilization was generally unaffected during the study in any dosed group. Hematological examinations performed on 10 control rats and five rats from each test group immediately prior to termination at week 13 revealed normal values. At necropsy, the weights of the liver, kidneys, heart, lungs, testes, spleen, and thyroid and adrenal glands were recorded. Tissues from the above organs and the stomach, duodenum, ileum, caecum and colon were subsequently preserved in formalin for histopathologic examinations. Absolute and relative kidney weights were increased for the mid- and high-dose groups; however, these changes were not accompanied by any evidence of histopathology. Other tissues examined were also within normal limits. The no-observed-adverse-effect level (NOAEL) was concluded to be 50 mg/kg body weight per day, based on significantly reduced body weight gain at the high-dose level of 8200 ppm (410 mg/kg/day) (Wheldon et al., 1967). The intake level 50 mg/kg body weight per day is at least 100,000 times the daily per capita intake¹ (“eaters only”) of 5-methyl-6,7-dihydro-5H-cyclopentapyrazine from use as a flavoring substance.

1.4.2.2. Oxygenated pyrazine derivatives

1.4.2.2.1. Acetylpyrazine (No. 24), methoxypyrazine (No. 28) and (2 or 5 or 6)-methoxy-3-methylpyrazine (No. 29). In separate 13-week dietary studies, 16 male and 16 female Sprague–Dawley rats (CD strain) were maintained on diets calculated to provide an average daily intake of 8.25 (M) and 8.15 (F) mg/kg body weight per day of acetylpyrazine (No. 24) or 45 (M) and 53 (F) mg/kg body weight per day of (2 or 5 or 6)-methoxy-3-methylpyrazine. Control animals were given a basic diet. The study protocols were described above (Posternak et al., 1975, 1969). Based on measurements of growth rate and food intake, hematological examinations, clinical chemistry determinations, organ weights and gross and histopathological examination, no differences were observed between test and control animals (Posternak et al., 1969, 1975). The intake levels 8.25 (M) and 8.15 (F) mg/kg body weight per day or 45 (M) and 53 (F) mg/kg body weight per day of (2 or 5 or 6)-methoxy-3-methylpyrazine are at least 1000 times the daily per capita intake¹ (“eaters only”) of acetylpyrazine or (2 or 5 or 6)-methoxy-3-methylpyrazine, respectively from use as a flavoring ingredient.

A control (24/sex) and three test groups (16/sex/group at low dose; 12/sex/group at mid-dose; 10/sex/group at high dose) of the CD strain of Sprague–Dawley albino

male and female rats were maintained individually in temperature- and humidity-controlled housing with ad lib. access to water and food. Test groups were maintained on diets containing methoxypyrazine (No. 28) at levels calculated to provide an average daily intake of 20, 63 or 200 mg/kg body weight for a period of 13 weeks. The concentration of the test material in the diet was adjusted weekly to maintain a constant level.

Clinical observations made twice daily showed no sign of obvious systemic toxicity in test groups of animals. Weekly measurements of food consumption, body weights and efficiency of food utilization revealed decreased mean body weights for males and females at the 200 mg/kg body weight per day level and for females at the 63 mg/kg body weight per day level, but only during week 13 of the study. These same groups also consumed significantly less food than the controls. No difference in efficiency of food utilization was recorded between test and control groups. Hematological examinations, clinical chemistry determinations and urine analyses performed during weeks 6 and 12 of the study revealed normal values. All animals were killed after 13 weeks. All animals were subjected to detailed necropsy examination and liver, heart, testes, ovaries and kidney weights were measured. Thyroid and adrenal lobes were weighed after fixation. All tissues and organs from each animal were preserved and histopathological examinations were performed on hematoxylin and eosin-stained sections of the major organs and tissues. Minor increases in absolute and relative liver weight were reported in the high dose of both sexes. However, there was no evidence of liver histopathology (Osborne et al., 1981). The NOAEL of 20 mg/kg body weight per day in rats is greater than 100,000 times the daily per capita intake¹ (eaters only) of 1×10^{-5} mg/kg body weight from use of methoxypyrazine as a flavoring substance.

1.4.2.3. Alkylpyrazines containing a thiol or sulfide function

1.4.2.3.1. 2-Pyrazinylethanethiol (No. 36), pyrazinylmethyl methyl sulfide (No. 37) and (3 or 5 or 6)-(methylthio)-2-methylpyrazine (No. 38). Each of these pyrazine derivatives has been studied in a 90-day rat dietary safety evaluation study using a protocol previously described (Posternak et al., 1975). 2-pyrazinylethanethiol, (3 or 5 or 6)-(methylthio)-2-methylpyrazine, and pyrazinylmethyl methyl sulfide were incorporated into the diets of rats at levels calculated to provide an average daily intake of 16.3, 4.0 and 1.6 mg/kg body weight, respectively.

Slight (<10%) statistically significant reductions in body weight gain were accompanied by a slight decrease in efficiency of food utilization in male animals fed (3 or 5 or 6)-(methylthio)-2-methylpyrazine. In the absence of any other evidence of toxicity, the authors concluded that these changes were of no biological significance and a result of poor palatability. Male rats fed 16.3 mg

2-pyrazinylethanethiol/kg body weight per day showed slight (<10%) increases in absolute and relative kidney weights, but these minimal changes were not accompanied by any evidence of substance-related histopathology, and were therefore concluded by the authors to be of no toxicological significance. No differences were observed between test and control animals maintained on diets containing 1.6 mg pyrazinylmethyl methyl sulfide/kg body weight per day (Posternak et al., 1975). The intake level of 16.3 mg 2-pyrazinylethanethiol/kg body weight per day, 4.0 mg (3 or 5 or 6)-(methylthio)-2-methylpyrazine/kg body weight per day, and 1.6 mg pyrazinylmethyl methyl sulfide/kg body weight per day is at least 1000 times the daily per capita intake¹ ("eaters only") from use of each of the three pyrazine derivatives as flavoring substances.

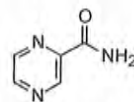
1.4.3. Carcinogenicity study with a structurally-related pyrazine

1.4.3.1. Mice

Pyrazine-2-carboxylic acid derivatives and 5-hydroxypyrazine-2-carboxylic acid derivatives are major urinary metabolites formed by side-chain oxidation and ring hydroxylation of alkyl-substituted pyrazine derivatives discussed in this monograph. A structurally-related pyrazine derivative, the antitubercular drug, pyrazinamide² has been shown to hydrolyze to pyrazine-2-carboxylic acid in humans and laboratory animals (Weiner and Tinker, 1972). The ring hydroxylated metabolite 5-hydroxypyrazine-2-carboxylic acid has been identified as a metabolite of pyrazine-2-carboxylic acid in animals. Therefore, data for this substance are considered relevant and have been included in the discussion.

In a carcinogenicity study, groups of 35 male or 35 female 42-day-old B6C3F1 mice were provided with pyrazinamide³ (the amide derivative of pyrazine-2-carboxylic acid) in the diet at concentrations of 5000 or 10,000 ppm, 5 days per week for a period of 78 weeks (NCI, 1977). These dietary concentrations were calculated (FDA, 1993) to provide corresponding average daily intake levels of 750 or 1500 mg/kg body weight per day, respectively. Matched controls consisted of groups of 15 untreated mice per sex. The animals were observed twice a day for signs of toxicity throughout the treatment period and for an additional 26 or 27 weeks post-treatment. Body weights were measured once every 2

²



³ Pyrazinamide differs from nicotinamide by the replacement of the pyridine ring with a pyrazine ring. It is used in the treatment of tubercular infections when other drugs are ineffective and has been associated with liver damage. It is used in combination with other drugs at daily dose levels not greater than 3 grams (NCI, 1977).

weeks for the first 20 months, and once per month thereafter. After day 100, all moribund animals were killed and necropsied. Gross and microscopic evaluations were performed on all major tissues, major organs, and gross lesions of animals that died or were killed.

Mean body weights of treated animals were higher than controls from week 25 to the end of the study for males, equal to or higher than controls for high-dose females, and lower than controls for low-dose females. The authors noted that fluctuations of the growth curve might be associated with mortality rather than treatment with the test material. In male mice, there was no statistically significant dose-related trend in mortality [controls, 9/15 (60%); low-dose, 33/35 (94%); high-dose, 29/35 (83%)]. In female mice, there was a statistically significant increase in survival with increasing dose [controls, 9/15 (60%); low dose, 24/35 (69%); high-dose, 31/35 (89%)]. The authors reported that eight low-dose females escaped during the period of 10–39 weeks.

Non-neoplastic lesions were observed in both sexes of treated and control animals, but most were associated with aging. Interstitial and suppurative myocarditis was associated with increased deaths of treated animals. Suppurative bronchopneumonia and tracheitis also were attributed to increased mortality of both sexes of treated and control animals. Some neoplastic lesions were observed in treated and control male mice, but there was no statistically significant, dose-related trend. The increased incidence of lymphoma in female mice [controls, 0/13 (0%); low-dose, 2/25 (8%); high-dose, 6/29 (21%)] was statistically significant in the Cochran–Armitage test, but not the Fisher exact test. The Cochran–Armitage test looks at dose-related trends while the Fisher exact test is a direct comparison of treated groups and matched control groups. The authors noted that the increased incidence of lymphoma might be associated with decreased survival of female controls. Therefore, based on the small size and poor survival of the female control group, the association of lymphoma with administration of test material in female mice was deemed equivocal. No other statistically significant dose-related neoplasms were observed in female mice. The authors concluded that pyrazinamide is not carcinogenic in male B6C3F1 mice at dose levels of 750 or 1500 mg/kg body weight per day under conditions of the study, but the carcinogenicity of the substance in female B6C3F1 mice could not be fully evaluated (NCI, 1977).

1.4.3.2. Rats

In a carcinogenicity study, groups of 35 male or 35 female 42-day-old Fischer 344 rats were provided pyrazinamide in the diet at concentrations of 5000 or 10,000 ppm, 5 days per week for a period of 78 weeks. These dietary concentrations were calculated (FDA, 1993) to provide corresponding average daily intake

levels of 500 or 1000 mg/kg body weight per day, respectively. The test protocol was the same as that used in the 2-year mouse study above.

Mean body weights of treated animals were slightly lower than the controls for male rats and similar to controls for female rats. Overall survival was high, and there was no statistically significant dose-related trend in mortality for either male or female rats [males: controls, 11/15 (73%); low-dose, 29/35 (83%); high-dose, 30/36 (83%); females: controls, 13/15 (87%); low-dose, 21/35 (60%); high-dose, 29/34 (85%)]. Non-neoplastic lesions, typically associated with aging, were observed in both sexes of treated and control animals.

Some neoplastic lesions were observed in treated and control male rats, but there was no statistically significant, dose-related trend. A statistically significant (Cochran–Armitage test, $P=0.037$) decrease in the incidence of leukemia in treated groups was reported compared to that for the control groups. An increase in the incidence of pituitary chromophobe adenomas and carcinomas was observed in low-dose (43 and 3%, respectively) and high-dose (29 and 0%, respectively) females as compared to controls (14 and 0%, respectively), but the combined incidences were not dose related and not statistically significant. The authors concluded that under conditions of the study pyrazinamide was not carcinogenic in male and female F344 rats at dose levels of 500 or 1000 mg/kg body weight per day (NCI, 1977).

1.4.4. Genotoxicity studies

Genotoxicity testing has been performed on eight representative substances in this group. The results of these tests are summarized in Table 3 and described below.

1.4.4.1. *In vitro*

Nine pyrazine derivatives (Nos 1, 2, 5, 6, 7, 11, 14, 29 and 40) have been tested in the Ames assay with uniformly negative results up to concentrations of 3600 µg/plate in various strains of *Salmonella typhimurium* (SAL) with and without S9 metabolic activation (Stich et al., 1980; Wild et al., 1983; Aeschbacher et al., 1989; Lee et al., 1994). In a single study, 2-methylpyrazine, 2-ethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine and pyrazine have been tested in mitotic cross-over-gene conversion in *Saccharomyces cerevisiae* and chromosome aberrations in Chinese hamster ovary (CHO) cells (Stich et al., 1980). Cultures of stationary phase *S. cerevisiae* strain D5 showed an increase in the percentage aberrations among survivors at test concentrations ranging from 3300 to 135,000 µg/ml. However, there was no absolute increase in the number of aberrations compared to controls. Therefore, the increased percentages represent an artifact produced by

Table 3
In vitro genotoxicity studies for pyrazine derivatives used as flavoring substances

Agent	Test system	Test object	Concentration of agent	Results	Reference
1. 2-Methylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA102	0.94–94,000 µg/plate	Negative ^a	Aeschbacher et al. (1989)
1. 2-Methylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100	Not reported	Negative	Lee et al. (1994)
1. 2-Methylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA1537	6300–100,000 µg/plate	Negative ^a	Stich et al. (1980)
1. 2-Methylpyrazine	Mutation assay	<i>S. cerev.</i> strain D5	8500–67,500 µg/ml	Positive ^b	Stich et al. (1980)
1. 2-Methylpyrazine	Chrom. abs.	CHO cells	25,000–40,000	Positive ^a	Stich et al. (1980)
2. 2-Ethylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA102	2,500–20,000 µg/ml	Positive ^a	Aeschbacher et al. (1989)
2. 2-Ethylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA1537	0.97–97,200 µg/plate	Negative ^a	Stich et al. (1980)
2. 2-Ethylpyrazine	Mutation assay	<i>S. cerev.</i> strain D5	6300–100,000 µg/plate	Negative ^a	Stich et al. (1980)
2. 2-Ethylpyrazine	Chrom. abs.	CHO cells	8500–67,500 µg/ml	Positive ^b	Stich et al. (1980)
2. 2-Ethylpyrazine	Chrom. abs.	CHO cells	5000	Positive ^a	Stich et al. (1980)
5. 2,3-Dimethylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA102	2500 µg/ml	Positive ^a	Aeschbacher et al. (1989)
5. 2,3-Dimethylpyrazine	Ames test	<i>S. typh.</i> TA 98, TA 100	0.97–97,200 µg/plate	Negative ^a	Lee et al. (1994)
6. 2,5-Dimethylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA102	Not reported	Negative ^a	Aeschbacher et al. (1989)
6. 2,5-Dimethylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100	0.97–97,200 µg/plate	Negative ^a	Lee et al. (1994)
6. 2,5-Dimethylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA1537	Not reported	Negative ^a	Stich et al. (1980)
6. 2,5-Dimethylpyrazine	Mutation assay	<i>S. cerev.</i> strain D5	12,500–200,000 µg/plate	Positive ^b	Stich et al. (1980)
6. 2,5-Dimethylpyrazine	Chrom. abs.	CHO cells	16,900–135,000 µg/ml	Positive ^b	Stich et al. (1980)
7. 2,6-Dimethylpyrazine	Ames test	<i>S. typh.</i> TA100, TA98	25,000–40,000	Positive ^a	Stich et al. (1980)
7. 2,6-Dimethylpyrazine	Ames test	<i>S. typh.</i> TA100, TA98	2500–20,000 µg/ml	Positive ^a	Lee et al. (1994)
7. 2,6-Dimethylpyrazine	Ames test	<i>S. typh.</i> TA98	86–10,800	Negative ^a	Lee et al. (1994)
7. 2,6-Dimethylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA102	2160–10,800	Positive ^b	Aeschbacher et al. (1989)
7. 2,6-Dimethylpyrazine	Mutation assay	<i>S. typh.</i> TA98, TA100, TA1537	86–10,800 µg/plate	Negative ^a	Stich et al. (1980)
7. 2,6-Dimethylpyrazine	Chrom. abs.	<i>S. cerev.</i> strain D5	0.54–54,000 µg/plate	Negative ^a	Stich et al. (1980)
7. 2,6-Dimethylpyrazine	Chrom. abs.	CHO cells	6300–100,000 µg/plate	Positive ^b	Stich et al. (1980)
11. 2,3-Diethylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA102	3300–33,800 µg/ml	Positive ^a	Stich et al. (1980)
14. 2,3,5-Trimethylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA102	5000–10,000	Positive ^a	Aeschbacher et al. (1989)
29. (2, 5 or 6)-Methoxy-3-methylpyrazine	Ames test	<i>S. typh.</i> Strains TA98, TA100, TA1535, TA1537, TA1538	2500 µg/ml	Positive ^a	Aeschbacher et al. (1989)
29. (2, 5 or 6)-Methoxy-3-methylpyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA102	1.08–109,000 µg/plate	Negative ^a	Aeschbacher et al. (1989)
29. (2, 5 or 6)-Methoxy-3-methylpyrazine	Ames test	<i>S. typh.</i> Strains TA98, TA100, TA1535, TA1537, TA1538	0.98–97,735 µg/plate	Negative ^a	Aeschbacher et al. (1989)
29. (2, 5 or 6)-Methoxy-3-methylpyrazine	Basal test	<i>Drosophila</i>	up to 3600 µg/plate	Negative ^a	Wild et al. (1983)
29. (2, 5 or 6)-Methoxy-3-methylpyrazine	Micronucleus assay	Mouse	10 mm	Negative	Wild et al. (1983)
40. Pyrazine	Ames test	<i>S. typh.</i> TA98, TA100, TA102	87–248 mg/kg	Negative	Wild et al. (1983)
40. Pyrazine	Ames test	<i>S. typh.</i> TA98, TA100	0.64–64,000 µg/plate	Negative ^a	Aeschbacher et al. (1989)
40. Pyrazine	Ames assay	<i>S. typh.</i> TA98, TA100, TA1537	Not reported	Negative ^a	Lee et al. (1994)
40. Pyrazine	Mutation	<i>S. cerev.</i> strain D5	6300–100,000 µg/plate	Negative ^a	Stich et al. (1980)
40. Pyrazine	Chrom. abs.	CHO cells	7500–60,000 µg/ml	Positive ^b	Stich et al. (1980)
40. Pyrazine	Chrom. abs.	CHO cells	5000–10,000 µg/ml	Positive ^a	Stich et al. (1980)

^a With and without metabolic activation.

^b Without metabolic activation.

^c With metabolic activation.

adjusting for reduced survival. No increase in the number of mitotic recombinants was observed in any test culture.

The unsubstituted and alkyl-substituted pyrazine derivatives all induced significant percentages of chromosome aberrations (breaks and exchanges) in metaphase plates in CHO cells with and without S9 activation at test concentrations ranging from 2500 to 40,000 µg/ml. However, the value of these results must be considered in the context that: (1) the percentage of metaphase plates with chromosomal aberrations were dose related, occurring only at concentrations that were two to four times less than those producing cytotoxicity; (2) the high concentrations 10,000 to 40,000 µg/ml of the weakly basic pyrazines may have altered cellular homeostasis; and (3) there were no negative controls upon which to demonstrate whether a significant increase in aberrations had actually occurred under conditions of the assay (Stich et al., 1980).

1.4.4.2. *In vivo*

(2 or 5 or 6)-Methoxy-3-methylpyrazine (No. 29) showed no evidence of mutagenicity when *Drosophila* were exposed to a concentration of 10 mM (140 µg/ml) (Wild et al., 1983).

In a mouse micronucleus test, male and female NMRI mice were treated once with an oral dose of 87, 174 or 248 mg (2 or 5 or 6)-methoxy-3-methylpyrazine/kg body weight. Animals were euthanized and bone marrow smears prepared 30 h after treatment. There was no evidence of an increase in micronuclei of bone marrow polychromatic erythrocytes (Wild et al., 1983).

1.4.4.3. *Conclusions on genotoxicity studies*

The relevance of positive results of *in vitro* assays with *S. cerevisiae* and CHO cells to human health assessments is questionable for the following reasons: (1) the studies were performed in a single study at near-toxic concentrations thousands of times greater than those that can be achieved in humans exposed to pyrazines as flavoring substances; (2) many genotoxicity assays (i.e. CHO chromosomal aberration assay) performed prior to 1985 did not adequately study the influence of cytotoxicity (e.g. effect of lysosomal breakdown) (Zajac-Kaye and Ts'o, 1984; Bradley et al., 1987) under physiological conditions and controlled ionic strength and pH conditions (Brusick, 1986; Heck et al., 1989); and (3) the positive *in vitro* results have not been confirmed by any standard *in vivo* assay (Wild et al., 1983).

1.4.5. *Other relevant studies*

1.4.5.1. *Reproduction/developmental studies*

2,5-Dimethylpyrazine (No. 6) is a urinary metabolite formed endogenously in female rodents (Novotny et al.,

1986). It has been suggested that 2,5-dimethylpyrazine decreases the overall success of reproduction in rodents that are housed in groups. The study designs reported here have a basic research orientation rather than the standardized protocol normally used for hazard identification and dose-response evaluation. The route of exposure (subcutaneous injection) and relatively high dose levels at which effects were seen (> 70 mg/kg body weight per day) make it difficult to apply the results to the safety assessment of flavoring substances.

The effects of 2,5-dimethylpyrazine on reproductive and accessory reproductive organs in female rats were studied (Yamada et al., 1992). Following subcutaneous administration to female Wistar rats aged 3–7 weeks of 100 mg/kg body weight one to two times daily, uterus weight was significantly decreased while ovary weight and serum levels of estradiol were unaffected. With 2,5-dimethylpyrazine pretreatment two times a day for 2 and 4 days, the uterine-weight increase normally observed in ovariectomized rats after estradiol injection was inhibited. The uptake of ³H-estradiol by the uterus was also significantly decreased by 2,5-dimethylpyrazine treatment. According to the authors, these results suggest that 2,5-dimethylpyrazine may have direct inhibitory action on the uterus of rats.

Groups of 4- (juvenile) or 6-week-old male Wistar rats were dosed sc once daily for a period of 2 weeks with 2,5-dimethylpyrazine at doses of 10, 30, 70 or 100, and 100 or 300 mg/kg body weight per day, respectively. There were no effects on plasma testosterone, polyamines or acid phosphatase in the juvenile rat prostate following reported dose levels of 10 or 30 mg/kg body weight per day. In the juvenile rats, decreased levels of plasma testosterone and prostate spermine were observed at ≥70 mg/kg body weight per day, and decreased levels of spermidine and acid phosphatase in the prostate were reported at ≥70 mg/kg body weight per day. However, these effects were not obtained on administration of the test substance to mature male rats at the same dose levels. The findings suggest that in juvenile rats a high dose of 2,5-dimethylpyrazine inhibits the biosynthesis of polyamines and acid phosphatase in the prostate by decreasing the circulating testosterone level (Yamada et al., 1994).

The effects of dimethylpyrazine isomers (2,3-dimethylpyrazine (No. 5), 2,5-dimethylpyrazine (No. 6) 2,6-dimethylpyrazine (No. 7) on reproductive and accessory reproductive organs were investigated in a study with male rats. Following daily sc administration of 100 mg 2,5-dimethylpyrazine/kg body weight for a period of 2 weeks, the weights of prostate and seminal vesicles, plasma testosterone levels, acid phosphatase activity in the prostate, and fructose content in the seminal vesicles all were decreased. Testis weight and testis acid phosphatase activity were not affected by 2,5-dimethylpyrazine, nor were numbers of spermatozoa in the

epididymis. At the same dose level, 2,6-dimethylpyrazine affected only the seminal vesicles, while 2,3-dimethylpyrazine had no influence on accessory reproductive organs. The authors concluded that 2,5-dimethylpyrazine induced decreased prostate and seminal vesicle weights by inhibiting testosterone uptake and reducing plasma testosterone levels (Yamada et al., 1993).

Four groups of 10 virgin Crl CD rats were administered 0, 25, 125 or 250 mg/kg body weight of tetramethylpyrazine (No. 20) by gavage once daily, 7 days prior to cohabitation, through cohabitation (maximum of 7 days), gestation, delivery, and a 4-day post-parturition period. Maternal indices monitored included twice-daily observation, measurement of body weights, food consumption, duration of gestation and fertility parameters (mating and fertility index, gestation index and number of offspring per litter). Offspring indices included daily observation, clinical signs, examination for gross external malformations, and measurement of body weight. The only effects reported included reduced body weight gain in the mid- and high-dose dams that was accompanied by a statistically significant reduction in food consumption in the high-dose group. There were no observed effects in the dams at the low dose or in the offspring at any dose level. The authors concluded that there were no reproductive or developmental effects (Vollmuth et al., 1990).

1.4.6. Special studies

A series of pyrazines [3, 5 or 6(methylthio)-2-methylpyrazine (No. 38), 6,7-dihydro-2,3-dimethyl-5*H*-cyclopentapyrazine (No. 22) and pyrazinylethanethiol (No. 36), have been investigated for potential hepatotoxicity and to assess whether they induce peroxisomal and/or microsomal enzyme activities (Beamand et al., 1992; Japenga et al., 1993). The authors reported that pyrazinylethanethiol was cytotoxic to primary rat hepatocyte cultures at concentrations greater than 0.2 mM, while the other compounds were cytotoxic only at much higher concentrations. None of the pyrazine derivatives induced palmitoyl-CoA oxidation (a marker for peroxisome proliferation), but they did induce cytochrome P-450-dependent enzymes.

Profiles of volatile metabolites of urine samples from normal individuals and subjects with diabetes mellitus have been studied by gas chromatography and mass spectrometry (Zlatkis et al., 1973). In normal subjects, pyrazines were minor constituents, but in subjects with diabetes mellitus under insulin treatment, high concentrations of pyrazines were found.

Tetramethylpyrazine (No. 20) was found to have inhibitory effects on platelet function. The anti-platelet activity of tetramethylpyrazine analogs was enhanced by an increased number of alkyl groups on the pyrazine ring as well as increased length of the unbranched alkyl

side-chains. Increased inhibition of platelet aggregation correlated with increased lipophilicity of the test substances (Liu and Sylvester, 1994).

1.5. Recognition of GRASr status

The group of pyrazine derivatives discussed here was determined to be generally recognized as safe (GRAS) under conditions of intended use as flavor ingredients by the FEMA Expert Panel in 1965. In 1976, the Panel evaluated the available data and affirmed the GRAS status of this flavor ingredient (GRASa). In 1993, the Panel initiated a comprehensive program to reevaluate the status of all FEMA GRAS flavor ingredients concurrent with a systematic revision of the FEMA Scientific Literature Reviews (SLRs). The group of pyrazine derivatives was reaffirmed as GRAS (GRASr) based, in part, on their extremely low aroma thresholds and their self-limiting properties in food; their rapid absorption, metabolic detoxication and excretion in humans; their low level of flavor use; the wide margins of safety between the conservative estimates of intake and the no-adverse-effect levels determined from subchronic and chronic studies and the lack of genotoxic and mutagenic potential. This evidence of safety is supported by the intake of pyrazine derivatives as natural components of traditional foods is much greater than their intake as intentionally added flavoring substances.

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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 50, Revision 1 (FGE.50Rev1):

Consideration of pyrazine derivatives evaluated by JECFA (57th meeting) structurally related to pyrazine derivatives evaluated by EFSA in FGE.17Rev2 (2010)¹

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)^{2,3}

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ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to consider evaluations of flavouring substances assessed since 2000 by the Joint FAO/WHO Expert Committee on Food Additives (the JECFA), and to decide whether further evaluation is necessary, as laid down in Commission Regulation (EC) No 1565/2000. Since the previous version of FGE.50, new *in vitro* and *in vivo* genotoxicity data on 5-methylquinoxaline [FL-no: 14.028] have been provided. The Panel concluded that these data allowed to rule out genotoxicity concerns for the substance. 5-Methylquinoxaline was then evaluated through a stepwise approach (the Procedure) that integrates information on structure-activity relationships, intake from current uses, toxicological threshold of concern, and available data on metabolism and toxicity. The Panel concluded that the substance do not give rise to safety concerns at the levels of dietary intake, estimated on the basis of the MSDI approach. So in total, for all the 41 JECFA evaluated pyrazines derivatives [FL-no: 14.005, 14.006, 14.015, 14.017, 14.018, 14.019, 14.020, 14.021, 14.022, 14.024, 14.025, 14.026, 14.027, 14.028, 14.031, 14.032, 14.034, 14.035, 14.037, 14.043, 14.044, 14.049, 14.050, 14.053, 14.054, 14.055, 14.056, 14.062, 14.067, 14.069, 14.077,

1 On request from the Commission, Question No EFSA-Q-2010-00007, adopted on 25 November 2010.

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14.082, 14.095, 14.096, 14.098, 14.100, 14.114, 14.121, 14.123, 14.142 and 14.144] evaluated in FGE.50, the Panel agrees with the JECFA conclusion, “No safety concern at estimated levels of intake as flavouring substances” based on the MSDI approach. Adequate specifications for the materials of commerce are available for all 41 flavouring substances.

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SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) to provide scientific advice to 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 requested to consider the Joint FAO/WHO Expert Committee on Food Additives (the JECFA) evaluations of flavouring substances assessed since 2000, and to decide whether no further evaluation is necessary, as laid down in Commission Regulation (EC) No 1565/2000. These flavouring substances are listed in the Register, which was adopted by Commission Decision 1999/217/EC and its consecutive amendments.

The present consideration concerns 41 pyrazine derivatives evaluated by the JECFA (57th meeting) and will be considered in relation to the EFSA evaluation of 21 pyrazine derivatives evaluated in the Flavouring Group Evaluation 17, Revision 2 (FGE.17Rev2).

The Panel concluded that the 41 substances in the JECFA flavouring group of pyrazines are structurally related to the pyrazines evaluated by EFSA in FGE.17Rev2.

In the previous version of the present Flavouring Group Evaluation (FGE), the Panel concluded that it could agree in the way the application of the Procedure has been performed by the JECFA for 40 out of the 41 pyrazines derivatives. For 5-methylquinoxaline [FL-no: 14.028], the Panel concluded that in line with the conclusions for quinoxaline and the two quinoxaline derivatives (quinoxaline [FL-no: 14.147], 2-methylquinoxaline [FL-no: 14.139] and 2,3-dimethylquinoxaline [FL-no: 14.108]) in FGE.17Rev1, 5-methylquinoxaline should not be evaluated using the Procedure until adequate genotoxicity data become available. New genotoxicity data have now become available and based on these data the Panel concluded that the *in vitro* genotoxicity alert could be ruled out for 5-methylquinoxaline [FL-no: 14.028] as no genotoxic potential at gene or chromosome level was indicated.

For all 41 substances evaluated through the JECFA Procedure intake data are available for EU.

For all 41 substances evaluated through the Procedure use levels are needed to calculate the mTAMDI in order to identify those flavouring substances that need more refined exposure assessment and to finalise the evaluation.

In order to determine whether the conclusion for the 41 JECFA evaluated substances can be applied to the materials of commerce, it is necessary to consider the available specifications. Adequate specifications including complete purity criteria and identity are available for all 41 JECFA evaluated substances.

For all the 41 JECFA-evaluated pyrazines [FL-no: 14.005, 14.006, 14.015, 14.017, 14.018, 14.019, 14.020, 14.021, 14.022, 14.024, 14.025, 14.026, 14.027, 14.028, 14.031, 14.032, 14.034, 14.035, 14.037, 14.043, 14.044, 14.049, 14.050, 14.053, 14.054, 14.055, 14.056, 14.062, 14.067, 14.069, 14.077, 14.082, 14.095, 14.096, 14.098, 14.100, 14.114, 14.121, 14.123, 14.142 and 14.144] the Panel

agrees with the JECFA conclusion, “No safety concern at estimated levels of intake as flavouring substances” based on the MSDI approach.

KEY WORDS

Pyrazines, JECFA 57th meeting, pyrazine derivatives, quinoxaline derivatives, FGE.17Rev2.

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BACKGROUND

Regulation (EC) No 2232/96 of the European Parliament and the Council (EC, 1996a) 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 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 2009/163/EC (EC, 2009a). 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, 1999a).

Commission Regulation (EC) No 1565/2000 lays down that substances that are contained in the Register and will be classified in the future by the Joint FAO/WHO Expert Committee on Food Additives (the JECFA) so as to present no safety concern at current levels of intake will be considered by the European Food Safety Authority (EFSA), who may then decide that no further evaluation is necessary.

In the period 2000 – 2008, during its 55th, 57th, 59th, 61st, 63rd, 65th, 68th and 69th meetings, the JECFA evaluated about 1000 substances, which are in the EU Register.

TERMS OF REFERENCE

EFSA is requested to consider the JECFA evaluations of flavouring substances assessed since 2000, and to decide whether no further evaluation is necessary, as laid down in Commission Regulation (EC) No 1565/2000 (EC, 2000a). These flavouring substances are listed in the Register which was adopted by Commission Decision 1999/217 EC (EC, 1999a) and its consecutive amendments.

In addition, in letter of 1 April 2009 the Commission requested EFSA to carry out a re-evaluation of flavouring substances [FL-no: 14.028, 14.108 and 14.139] in accordance with Commission Regulation (EC) No 1565/2000:

“The European Commission requests the European Food Safety Authority to carry out a risk assessment on 5-methylquinoxaline ([FL-no: 14.028]), 2,3-dimethylquinoxaline ([FL-no: 14.108]) and 2-methylquinoxaline ([FL-no: 14.139]) in accordance with Commission Regulation (EC) No 1565/2000, if possible by the end of the evaluation programme, if not within nine month from finalisation of that programme”.

The deadline of the Terms of Reference was negotiated to 30 June 2010.

ASSESSMENT

The approach used by EFSA for safety evaluation of flavouring substances is referred to in Commission Regulation (EC) No 1565/2000 (EC, 2000a), hereafter named the “EFSA Procedure”. This Procedure is based on the Opinion of the Scientific Committee on Food (SCF, 1999a), which has been derived from the evaluation procedure developed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1995; JECFA, 1996a; JECFA, 1997a; JECFA, 1999b), hereafter named the “JECFA Procedure”. The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) compares the JECFA evaluation of structurally related substances with the result of a corresponding EFSA evaluation, focussing on specifications, intake estimations and toxicity data, especially genotoxicity data. The evaluations by EFSA will conclude whether the flavouring

substances are of no safety concern at their estimated levels of intake, whether additional data are required or whether certain substances should not be put through the EFSA Procedure.

The following issues are of special importance.

Intake

In its evaluation, the Panel as a default uses the “Maximised Survey-derived Daily Intake” (MSDI) approach to estimate the *per capita* intakes of the flavouring substances in Europe.

In its evaluation, the JECFA includes intake estimates based on the MSDI approach derived from both European and USA production figures. The highest of the two MSDI figures is used in the evaluation by the JECFA. It is noted that in several cases, only the MSDI figures from the USA were available, meaning that certain flavouring substances have been evaluated by the JECFA only on the basis of these figures. For Register substances for which this is the case the Panel will need EU production figures in order to finalise the evaluation.

When the Panel examined the information provided by the European Flavour Industry on the use levels in various foods, it appeared obvious that the MSDI approach in a number of cases would grossly underestimate the intake by regular consumers of products flavoured at the use level reported by the Industry, especially in those cases where the annual production values were reported to be small. In consequence, the Panel had reservations about the data on use and use levels provided and the intake estimates obtained by the MSDI approach. It is noted that the JECFA, at its 65th meeting considered “how to improve the identification and assessment of flavouring agents, for which the MSDI estimates may be substantially lower than the dietary exposures that would be estimated from the anticipated average use levels in foods” (JECFA, 2006c).

In the absence of more accurate information that would enable the Panel to make a more realistic estimate of the intakes of the flavouring substances, the Panel has decided also to perform an estimate of the daily intakes per person using a “modified Theoretical Added Maximum Daily Intake” (mTAMDI) approach based on the normal use levels reported by Industry.

As information on use levels for the flavouring substances has not been requested by the JECFA or has not otherwise been provided to the Panel, it is not possible to estimate the daily intakes using the mTAMDI approach for the substances evaluated by the JECFA. The Panel will need information on use levels in order to finalise the evaluation.

Threshold of 1.5 Microgram/Person/Day (Step B5) Used by the JECFA

The JECFA uses the threshold of concern of 1.5 microgram/person/day as part of the evaluation procedure:

“The Committee noted that this value was based on a risk analysis of known carcinogens which involved several conservative assumptions. The use of this value was supported by additional information on developmental toxicity, neurotoxicity and immunotoxicity. In the judgement of the Committee, flavouring substances for which insufficient data are available for them to be evaluated using earlier steps in the Procedure, but for which the intake would not exceed 1.5 microgram per person per day would not be expected to present a safety concern. The Committee recommended that the Procedure for the Safety Evaluation of Flavouring Agents used at the forty-sixth meeting be amended to include the last step on the right-hand side of the original procedure (“Do the condition of use result in an intake greater than 1.5 microgram per day?”) (JECFA, 1999b).

In line with the Opinion expressed by the Scientific Committee on Food (SCF, 1999), the Panel does not make use of this threshold of 1.5 microgram per person per day.

Genotoxicity

As reflected in the Opinion of SCF (SCF, 1999a), the Panel has in its evaluation focussed on a possible genotoxic potential of the flavouring substances or of structurally related substances. Generally, substances for which the Panel has concluded that there is an indication of genotoxic potential *in vitro*, will not be evaluated using the EFSA Procedure until further genotoxicity data are provided. Substances for which a genotoxic potential *in vivo* has been concluded, will not be evaluated through the Procedure.

Specifications

Regarding specifications, the evaluation by the Panel could lead to a different opinion than that of JECFA, since the Panel requests information on e.g. isomerism.

Structural Relationship

In the consideration of the JECFA evaluated substances, the Panel will examine the structural relationship and metabolism features of the substances within the flavouring group and compare this with the corresponding FGE.

HISTORY OF THE EVALUATION OF THE SUBSTANCES IN THE PRESENT FGE

In FGE.50, which contain a group of 41 flavouring substances consisting of pyrazine and pyrazine derivatives, the Panel concluded that for one of these substances, 5-methyl quinoxaline [FL-no: 14.028], the Procedure should not be applied until adequate genotoxicity data become available. This conclusion was in line with the Panel conclusion for three other quinoxalines evaluated in FGE.17Rev1 (EFSA, 2008r).

FGE	Opinion adopted by EFSA	Link	No. of candidate substances
FGE.50	February 2007	http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178692189146.htm	41
FGE.50Rev1	November 2010		41

Additional genotoxicity data have now become available for [FL-no: 14.028] and the present revision of FGE.50, FGE.50Rev1 includes the evaluation of these genotoxicity data submitted by the Industry (Flavour Industry, 2009a).

Of the 41 flavouring substances considered by EFSA in FGE.50 no European production volumes were available for seven substances [FL-no: 14.025, 14.026, 14.034, 14.067, 14.069, 14.077 and 14.121]. As no European production volumes were available no MSDI could be calculated for EU and accordingly the substances could not be considered by EFSA using the Procedure. In the course of 2010, Industry provided EU production figures (EFFA, 2010a; EFFA, 2010c) for these seven substances together with similar data on approximately 100 other substances from 27 different FGEs. In order to avoid unnecessary delay, these substances were evaluated in a special FGE, FGE.96, in which EU production volumes / anticipated production volumens submitted on request by DC SANCO have been included in the evaluation (EFSA, 2010aj). The EU production volumes of these seven substances and the outcome of the evaluations have also been included in the current revision of FGE.50 (EFFA, 2010a; EFFA, 2010c).

Finally, since the previous version of FGE.50 (EFFA, 2010a), missing information have been provided on the stereoisomeric composition for two substances [FL-no: 14.037 and 14.062], identity test for one substance [FL-no: 14.024], clarification of the specific gravity for three substances [FL-no: 14.019,

14.054 and 14.062] and composition of mixtures of positional isomers [FL-no: 14.006, 14.020, 14.021, 14.025, 14.035, 14.050, 14.055, 14.067, 14.077, 14.100, 14.114 and 14.121].

1. Presentation of the Substances in the JECFA Flavouring Group

1.1. Description

1.1.1. JECFA Status

The JECFA has evaluated a group of 41 flavouring substances consisting of pyrazine and pyrazine derivatives.

1.1.2. EFSA Considerations

The Panel concluded that all the 41 substances in the JECFA flavouring group of pyrazines are structurally related to the group of 21 pyrazines evaluated by EFSA in the Flavouring Group Evaluation 17, Revision 2 (FGE.17 Rev2).

1.2. Isomers

1.2.1. Status

Two of the JECFA evaluated substances [FL-no: 14.037 and FL-no: 14.062] have a chiral centre. Further 12 substances consist of mixtures of positional isomers [FL-no: 14.006, 14.020, 14.021, 14.025, 14.035, 14.050, 14.055, 14.067, 14.077, 14.100, 14.114 and 14.121].

1.2.2. EFSA Considerations

Industry has informed that the two substances [FL-no: 14.037 and FL-no: 14.062] occur as a racemic mixture (EFFA, 2010a).

Industry has informed about the composition of the mixture of positional isomers of the 12 JECFA evaluated substances [FL-no: 14.006, 14.020, 14.021, 14.025, 14.035, 14.050, 14.055, 14.067, 14.077, 14.100, 14.114 and 14.121] (EFFA, 2010a).

1.3. Specifications

1.3.1. JECFA Status

The JECFA specifications are available for all 41 substances (JECFA, 2001c). See Table 1.

1.3.2. EFSA Considerations

The available specifications are considered adequate for all 41 JECFA evaluated substances.

2. Intake Estimations

2.1. JECFA Status

For all 41 substances evaluated through the JECFA Procedure intake data are available for EU, see Table 3.1.

2.2. EFSA Considerations

No comments.

3. Genotoxicity Data

3.1. Genotoxicity Studies – Text Taken⁴ from the JECFA (JECFA, 2002a)

In vitro

2-Methylpyrazine [FL-no: 14.027], 2-ethylpyrazine [FL-no: 14.022], 2,3-dimethylpyrazine [FL-no: 14.050], 2,5-dimethylpyrazine [FL-no: 14.020], 2,6-dimethylpyrazine [FL-no: 14.021], 2,3,5-trimethylpyrazine [FL-no: 14.019], pyrazine [FL-no: 14.144]: These substances have been tested for their ability to cause reverse mutation, with uniformly negative results up to concentrations of 1000 microgram/plate in various strains of *Salmonella typhimurium* with and without an exogenous metabolic activation system from rodent liver (Stich et al., 1980; Aeschbacher et al., 1989; Lee et al., 1994a). In one of these studies, 2-methylpyrazine, 2-ethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, and pyrazine were also tested for their ability to cause mitotic crossover-gene conversion in *Saccharomyces cerevisiae* and chromosomal aberrations in Chinese hamster ovary cells (Stich et al., 1980). Surviving cells in cultures of stationary phase *S. cerevisiae* strain D5 showed an increase in the percentage of aberrant colonies at test concentrations of 3300–135 000 microgram/ml; however, no increase in the number of mitotic recombinants was observed among the aberrant colonies.

Pyrazine and the other alkyl-substituted pyrazine derivatives that were tested induced significant percentages of chromosomal aberrations (breaks and exchanges) in metaphase plates in Chinese hamster ovary cells with and without metabolic activation at test concentrations of 2500–40000 microgram/ml. However, these concentrations were two to four times lower than those that were cytotoxic, and no negative controls were used to allow a demonstration that a significant increase in the incidence of aberrations had actually occurred (Stich et al., 1980).

(2, 5 or 6)-Methoxy-3-methylpyrazine [FL-no: 14.025]: A mixture of three isomers, (2, 5 or 6)-methoxy-3-methylpyrazine, did not induce reverse mutation in *S. typhimurium* strain TA98, TA100, TA1535, TA1537, or TA1538 at concentrations of 3.6 mg/plate with and without metabolic activation (Wild et al., 1983).

In vivo

(2, 5 or 6)-Methoxy-3-methylpyrazine [FL-no: 14.025]: A test for Basc mutation was performed in *Drosophila* with a concentration of 10 mmol/L (140 microgram/ml), with no mutagenic effect (Wild et al., 1983).

⁴ The text is taken verbatim from the indicated reference source, but text related to substances not included in the present FGE has been removed.

Male and female NMRI mice were treated once orally with (2, 5, or 6)-methoxy-3-methylpyrazine at a dose of 87, 174, or 248 mg/kg bw and killed, and bone-marrow smears were prepared 30 h after treatment. There was no increase in the frequency of micronuclei in polychromatic erythrocytes (Wild et al., 1983).

Conclusion on genotoxicity

The relevance of the positive results for pyrazine and certain alkylpyrazines in assays with *S. cerevisiae* and Chinese hamster ovary cells *in vitro* reported by Stich et al. (1980) is unclear. The studies were performed at high, nearly toxic concentrations of the weakly basic pyrazines, which may have altered cellular homeostasis. The results of studies of genotoxicity by Zajac-Kaye & Ts'o (Zajac-Kaye & Ts'o, 1984), Brusick (Brusick, 1986), Bradley et al. (Bradley et al., 1987), and Heck et al. (Heck et al., 1989), for example, indicate that agents other than the pyrazines may have caused the observed results. The positive results *in vitro* reported by Stich et al. (Stich et al., 1980) were not corroborated by the results of studies conducted *in vivo* by Wild et al. (Wild et al., 1983) with (2, 5, or 6)-methoxy-3-methylpyrazine [FL-no: 14.025].

For a summary of *in vitro* / *in vivo* genotoxicity data considered by the JECFA see Table 2.1.

3.2. Genotoxicity Studies - Text Taken⁵ from EFSA FGE.17Rev2 (EFSA, 2010h)

Genotoxicity data were provided for three of the 20 candidate substances and for 11 of the 41 supporting substances. The three candidate substances are quinoxaline [FL-no: 14.147] and its derivatives 2-methylquinoxaline [FL-no: 14.139] and 2,3-dimethylquinoxaline [FL-no: 14.108]. After finalisation of the previous version of this FGE (i.e. FGE.17, Revision 1), both *in vitro* and *in vivo* genotoxicity data have become available for the supporting substance 5-methylquinoxaline [FL-no: 14.028]. This substance is a candidate flavouring substance in FGE.50. The Panel explored the option of using the genotoxicity data submitted for 5-methylquinoxaline [FL-no: 14.028] to support the evaluation of the genotoxic potential of the candidate quinoxaline derivatives in FGE.17.

Genotoxicity data on Candidate substances

In *in vitro* studies, quinoxaline [FL-no: 14.147], up to 10000 microgram/plate and 2,3-dimethylquinoxaline [FL-no: 14.108], up to 2500 microgram/plate, with and without metabolic activation, did not cause reverse mutation in various strains of *Salmonella typhimurium* (See Table IV.4). Two studies on 2-methylquinoxaline [FL-no: 14.139] are available, one study with a positive, the other with negative result in the Ames test. However, quinoxaline [FL-no: 14.147] at 250 microgram/ml culture medium and with metabolic activation was found to induce TFT-mutants in the mouse lymphoma mutagenesis assay (L5178Y TK^{+/−} cells). This study was conducted in accordance with the OECD guideline 476 and therefore considered valid.

No adequate *in vivo* studies on genotoxicity of the substances are available. A study of the potential of quinoxaline [FL-no: 14.147] to induce sperm head abnormalities (Topham, 1980) did not address a genetic endpoint and the Panel considered it could not be used for evaluation of genotoxicity of this substance.

Genotoxicity data on Supporting substances

Substituted pyrazines

⁵ The text is taken verbatim from the indicated reference source, but text related to substances not included in the present FGE has been removed.

In vitro, 2-methylpyrazine [FL-no: 14.027], ethylpyrazine [FL-no: 14.022], 2,3-dimethylpyrazine [FL-no: 14.050], 2,5-dimethylpyrazine [FL-no: 14.020], 2,3-diethylpyrazine [FL-no: 14.005], 2,6-dimethylpyrazine [FL-no: 14.021], 2,3,5-trimethylpyrazine [FL-no: 14.019], pyrazine [FL-no: 14.144] and (2, 5 or 6)-methoxy-3-methylpyrazine [FL-no: 14.025] were tested for their ability to cause reverse mutation in various strains of *S. typhimurium* and consistently revealed negative results with and without metabolic activation (Table 2.2).

In one of these studies, 2-methylpyrazine, ethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, and pyrazine were also tested for their potential to cause genotoxicity in *Saccharomyces cerevisiae* and chromosomal aberrations in Chinese hamster ovary cells (Table 2.2) (Stich et al., 1980). This study has strong limitations for the following reasons. The positive results were observed only in a narrow range of exceedingly high and toxic concentrations. In the case of chromosome aberrations, the concentration used exceeded the maximum level (5 mg/ml) recommended by OECD. It has been shown (Galloway, 2000) that *in vitro* chromosome breaking can occur secondary to toxicity and/or changed physiological conditions (e.g., pH, osmolarity) with compounds not able to react with DNA and negative in the Ames test and *in vivo*. The *S.cerevisiae* D5 assay for induction of "aberrant colonies" is not routinely used and has not been validated at international level due to the uncertainty on the various effects involved. Thus, the positive results reported by Stich et al. (Stich et al., 1980) are considered of limited value and not relevant for hazard and risk assessment. Furthermore, pyrazine was found negative in a wide range of concentrations both in the *Salmonella* assay and in the mouse lymphoma TK assay (Fung et al., 1988).

Quinoxalines

5-Methylquinoxaline [FL-no: 14.028] was examined for its mutagenic potential in *Salmonella typhimurium* strains TA100, TA1535, TA98 and TA1537, as well as in *Escherichia coli* strain WP2 uvrA. The study was conducted according to GLP and was in compliance with OECD Guideline 471. No evidence of mutagenicity was found with or without S9 metabolic activation at concentrations up to 5,000 µg/plate (Ogura & Wakamatsu, 2004).

5-methylquinoxaline was examined for its potential to induce structural chromosome aberrations in mammalian cells. The study was conducted according to GLP and was in compliance with OECD Guideline 473. The test system used was a subculture of Chinese hamster lung-derived CHL/IU cells that were exposed to the test material at concentrations of 320, 480 and 720 µg/mL without S9 mix, and 72.0, 228 and 720 µg/mL with S9 mix. The percentage of "cell productivity" (the cell number was measured and expressed as relative growth rate compared to negative control) was reported as a parameter for cytotoxicity. The Panel considered that 5-methylquinoxaline was found to induce chromosomal aberrations in cultured mammalian cells in the presence of metabolic activation. Additionally, an increased frequency of polyploid cells up to 12.5 % of the middle dose compared to 0 % in the control was observed in the presence and absence of metabolic activation at concentrations which induced only low cytotoxicity (Ajimu & Kawaguchi, 2004a).

In vivo data are available for two one of the supporting substances only, (2, 5 or 6)-Methoxy-3-methylpyrazine [FL-no: 14.025] and 5-methylquinoxaline [FL-no: 14.028].

5-Methylquinoxaline [FL-no: 14.028] was examined for its potential to induce micronucleated polychromatic erythrocytes (MNPCEs) in the bone marrow. The test material was administered daily (gavage) for two consecutive days to seven week old and six week old male SPF ICR (Crj:CD-1) mice at dosages of 125, 250 and 500 mg/kg/day (6 animals/dose). Microscopic examination of femoral bone marrow cells was conducted randomly from 5 animals. Two thousand polychromatic erythrocytes (PCE) per animal were analyzed microscopically (x1000), and the number of micronucleated polychromatic erythrocytes (MNCPE) was recorded. In order to evaluate the PCE/NCE ratio, the number of PCEs out of 200 total erythrocytes (PCEs plus NCEs) was recorded. The test was considered positive if the MNPCE frequencies in one or more treatment groups were significantly higher than that in the negative control groups. No significant increase of micronucleated

polychromatic erythrocyte frequency was observed in these treatment groups compared with the negative control group. The PCE/NCE ratio was not changed (Ajimu & Kawaguchi, 2004b). Based on the PCE/NCE ratio there is no indication that the substance reached the bone marrow, however, the Panel noted that the high dose was the maximum tolerated dose since clinical signs of toxicity have been observed after oral intake. Additionally, a doubling of this dose was lethal for two out of six animals in a preliminary test. Thus, the Panel considered it reasonable to assume that the substance was systemically available and reached the bone marrow.

For (2, 5 or 6)-methoxy-3-methylpyrazine [FL-no: 14.025] a test for Basc mutation was performed in *Drosophila* with a concentration of 10 mmol/L (140 microgram/ml) in the solutions/emulsions fed to the flies, with no mutagenic effect (Table 2.3). Secondly, male and female NMRI mice were treated once orally with 87, 174 or 248 mg/kg bw, bone-marrow smears were prepared only at one sampling time (at 30 hours) after treatment. There was no increase in the frequency of micronuclei in polychromatic erythrocytes (Table 2.3). The PCE/NCE ratio was not reported and thus, it is not clear if the test substance reached the bone marrow. However from this study, there is no evidence of genotoxic potential.

Conclusion on genotoxicity

The available data indicate that apparently there is no simple structure-activity relationship for the genotoxicity of quinoxalines, because the profile of genotoxic events *in vitro* differs for the various congeners (point mutations for [FL no: 14.139] and [FL-no: 14.147] vs chromosomal aberrations for [FL no: 14.028]). Therefore these compounds are to be evaluated based on substance-specific data for each individual quinoxaline derivative.

In vitro data indicate a genotoxic potential for quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139], for which no *in vivo* data are available. Therefore, for these two substances the Procedure cannot be applied until adequate genotoxicity data become available.

Conversely, 2,3-dimethylquinoxaline [FL-no: 14.108] is not considered genotoxic *in vitro* and hence can be evaluated through the Procedure (three negative bacterial reverse gene mutation assays which, although limited, consistently indicate lack of genotoxicity).

The Panel concluded that no genotoxic potential is indicated for 19 candidate substances, including 2,3-dimethylquinoxaline [FL-no: 14.108]. For these 19 substances, the available data do not preclude their evaluation through the Procedure.

For a summary of *in vitro* / *in vivo* genotoxicity data considered by EFSA see Table 2.2 and 2.3.

3.3. EFSA Considerations

The Panel considered that no genotoxic potential at gene or chromosome level is indicated for this group of flavourings.

4. Application of the Procedure

4.1. Application of the Procedure to Pyrazine and 40 Derivatives by JECFA (JECFA, 2002b)

According to the JECFA 32 of the substances belong to structural class II and nine to structural class III using the decision tree approach presented by Cramer *et al.* (Cramer et al., 1978).

The JECFA concluded all 41 pyrazines at step A3 in the JECFA Procedure – i.e. the substances are expected to be metabolised to innocuous products (step 2) and the intakes for all substances are below the thresholds for their structural classes II and III (step A3).

In conclusion, the JECFA evaluated all 41 substances as to be of no safety concern at the estimated levels of intake as flavouring substance based on the MSDI approach.

The evaluations of the 41 substances are summarised in Table 3.1: Summary of Safety Evaluation of Pyrazine and 40 Derivatives (JECFA, 2003a).

4.2. Application of the Procedure to 21 Pyrazine Derivatives by EFSA (FGE.17Rev2) (EFSA, 2010h)

In the previous version of FGE.17, FGE.17Rev1, it was found that two of the candidate substances, quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139] showed possible genotoxic potential *in vitro*. Therefore, the Panel decided that the Procedure could not be applied to these two candidate substances nor for the structurally related 2,3-dimethylquinoxaline [FL-no: 14.108] until adequate genotoxicity data become available.

Additional genotoxicity data have now become available for the structurally related 5-methylquinoxaline [FL-no: 14.028] and in the present FGE these genotoxicity data submitted by the Industry (Flavour Industry, 2009a) have been evaluated. The available data indicate that there is no apparent structure-activity relationship for the genotoxicity of quinoxalines (Hashimoto et al., 1979); thus these compounds are to be considered individually. 2,3-Dimethylquinoxaline [FL-no: 14.108] is not genotoxic *in vitro* and can be evaluated through the Procedure; conversely, *in vitro* data indicate a genotoxic potential for quinoxaline [FL-no: 14.147] and 2-methylquinoxaline [FL-no: 14.139], for which no *in vivo* data are available. Therefore, for these two substances the Procedure cannot be applied until adequate genotoxicity data become available.

Furthermore, the candidate substance, 2,5 or 6-methoxy-3-ethylpyrazine [FL-no: 14.051] no European production figures were available and consequently no European exposure estimates could be calculated. Accordingly, the safety in use could not be assessed using the Procedure for this substance.

Of the 18 candidate substances which are evaluated using the Procedure, 16 are classified into structural class II and two substances into structural class III using the decision tree approach presented by Cramer *et al.* (Cramer et al., 1978).

Sixteen of the 18 substances were concluded at step A3 – i.e. the substances are expected to be metabolised to innocuous products (step 2) and the estimated daily intakes are below the threshold for the structural class (step A3). Two candidate substances cannot be anticipated to be metabolised to innocuous products [FL-no: 14.122 and 14.052]. Therefore these two substances are evaluated along the B-side of the Procedure scheme.

For these substance the intake is below the threshold for the structural class (step B3). For 2-isopropyl-3-methyl thiopyrazine [FL-no: 14.122] a NOAEL exists to provide an adequate margin of safety to the estimated level of intake as flavouring substance (step B4). For one substance, isopropenylpyrazine [FL-no: 14.052] or for any relevant supporting substances no valid toxicity study from which a NOAEL could be established was available. Therefore, the Panel concluded that additional toxicity data are needed for this substance.

In conclusion the Panel considered that the 17 of the 18 substances evaluated through the Procedure were of no safety concern at the estimated levels of intake based on the MSDI approach.

The stepwise evaluations of the 18 substances are summarised in Table 3.2: Summary of Safety Evaluation Applying the Procedure (EFSA, 2010h).

4.3. EFSA Considerations

In the previous version of the present FGE, FGE50, the Panel concluded that they could agree in the way the application of the Procedure has been performed by the JECFA for 40 out of the 41 pyrazines derivatives. For 5-methylquinoxaline [FL-no: 14.028], the Panel concluded that in line with the conclusions for quinoxaline and the quinoxaline derivatives in FGE17.Rev1, 5-methylquinoxaline should not be evaluated using the Procedure until adequate genotoxicity data become available. Furthermore, for seven substances [FL-no: 14.025, 14.026, 14.034, 14.067, 14.069, 14.077 and 14.121], the evaluation could not be finalised due to missing EU production volumes.

New data on *in vitro* and *in vivo* genotoxicity for 5-methylquinoxaline [FL-no: 14.028] provided by Industry, were considered in this revision. Based on these data the Panel concluded that the *in vitro* genotoxicity alert could be ruled out for 5-methylquinoxaline [FL-no: 14.028] in FGE.50 as no genotoxic potential at gene or chromosome level was indicated.

Accordingly, the Panel therefore considered that no genotoxic potential is indicated for this group of 41 pyrazine derivatives.

The JECFA concluded at their 57th meeting that 5-methylquinoxaline [FL-no: 14.028] would be metabolised to innocuous products. The Panel agrees with the JECFA conclusion “no safety concern at estimated levels of intake as flavouring substances” based on the MSDI approach.

Based on newly submitted EU production volumes for the seven JECFA evaluated substances [FL-no: 14.025, 14.026, 14.034, 14.067, 14.069, 14.077 and 14.121] the MSDIs range from 0.0012 to 2.2 microgram/capita/day, which are all below the threshold for their respective structural class.

For all seven substances the Panel concluded at step A3 that these substances would be of no safety concern at their estimated level of intake based on the MSDI approach (EFSA, 2010aj).

5. Conclusion

The Panel concluded that the 41 substances in the JECFA flavouring group of pyrazines are structurally related to the pyrazines evaluated by EFSA in the Flavouring Group Evaluation 17 (FGE.17Rev2).

In the previous version of the present FGE, FGE50, the Panel concluded that it could agree in the way the application of the Procedure has been performed by the JECFA for 40 out of the 41 pyrazines derivatives. For 5-methylquinoxaline [FL-no: 14.028], the Panel concluded that in line with the conclusions for quinoxaline and the two quinoxaline derivatives (quinoxaline [FL-no: 14.147], 2-methylquinoxaline [FL-no: 14.139] and 2,3-dimethylquinoxaline [FL-no: 14.108]) in FGE17.Rev1, 5-methylquinoxaline should not be evaluated using the Procedure until adequate genotoxicity data become available. New genotoxicity data have now become available and based on these data the Panel concluded that the *in vitro* genotoxicity alert could be ruled out for 5-methylquinoxaline [FL-no: 14.028] as no genotoxic potential at gene or chromosome level was indicated.

For all 41 substances evaluated through the JECFA Procedure intake data are available for EU.

For all 41 substances evaluated through the Procedure use levels are needed to calculate the mTAMDI in order to identify those flavouring substances that need more refined exposure assessment and to finalise the evaluation.

In order to determine whether the conclusion for the 41 JECFA evaluated substances can be applied to the materials of commerce, it is necessary to consider the available specifications. Adequate specifications including complete purity criteria and identity are available for all the 41 JECFA evaluated substances.

For all the 41 JECFA evaluated pyrazines [FL-no: 14.005, 14.006, 14.015, 14.017, 14.018, 14.019, 14.020, 14.021, 14.022, 14.024, 14.025, 14.026, 14.027, 14.028, 14.031, 14.032, 14.034, 14.035, 14.037, 14.043, 14.044, 14.049, 14.050, 14.053, 14.054, 14.055, 14.056, 14.062, 14.067, 14.069, 14.077, 14.082, 14.095, 14.096, 14.098, 14.100, 14.114, 14.121, 14.123, 14.142 and 14.144] the Panel agrees with the JECFA conclusion, “No safety concern at estimated levels of intake as flavouring substances” based on the MSDI approach.

TABLE 1: SPECIFICATION SUMMARY

Table 1: Specifications Summary for the JECFA Evaluated Substances in the Present Group (JECFA, 2001c)

Table 1: Specification Summary of the Substances in the JECFA Flavouring Group of Pyrazines (JECFA, 2001c)

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)	EFSA comments
14.005 771	2,3-Diethylpyrazine		3136 534 15707-24-1	Liquid $C_8H_{12}N_2$ 136.20	Soluble Miscible	180 IR 97 %	1.492-1.509 0.956-0.976	
14.006 768	2-Ethyl-3-methylpyrazine		3155 548 15707-23-0	Liquid $C_7H_{10}N_2$ 122.17	Soluble Miscible	57 (13 hPa) IR 97 %	1.499-1.509 0.972-0.993	Mixture of positional isomers (2,3-; 2,5- and 2,6-isomers), 2,3- (75-85 %); 2,5- (15-25 %) and 2,6-isomers (1-2 %) (sum 97%) (EFFA, 2010a).
14.015 952	5,6,7,8-Tetrahydroquinoxaline		3321 721 34413-35-9	Solid $C_8H_{10}N_2$ 134.18	Moderately soluble Soluble	85 (4 hPa) 29-30 IR 98 %	n.a. n.a.	
14.017 770	2-Ethyl-5-methylpyrazine		3154 728 13360-64-0	Liquid $C_7H_{10}N_2$ 122.17	Soluble Miscible	79 (88 hPa) IR 95 %	1.491-1.501 0.960-0.970	
14.018 780	2,3,5,6-Tetramethylpyrazine		3237 734 1124-11-4	Solid $C_8H_{12}N_2$ 136.20	Slightly soluble Very soluble	190 85-90 IR 95 %	n.a. n.a.	
14.019 774	2,3,5-Trimethylpyrazine		3244 735 14667-55-1	Liquid $C_7H_{10}N_2$ 122.17	Soluble Miscible	171 IR 98 %	1.500-1.509 0.967-0.987	Specific gravity: depending on the quality and the producer, the SG ranges from: 0.967-0.987 (EFFA, 2010a).
14.020 766	2,5-Dimethylpyrazine		3272 2210 123-32-0	Liquid $C_6H_8N_2$ 108.14	Soluble Miscible	155 IR 98 %	1.497-1.503 0.982-1.000	Mixture of positional isomers (2,5- and 2,6-isomers), 2,5- (45-65 %) and 2,6-isomers (35-55 %) (sum 98 %) (EFFA, 2010a).
14.021 767	2,6-Dimethylpyrazine		3273 2211 108-50-9	Solid $C_6H_8N_2$ 108.14	Soluble Very soluble	154 48 IR 98 %	n.a. n.a.	Mixture of positional isomers (2,5- and 2,6-isomers), 2,5- (35-55 %) and 2,6-isomers (45-65 %) (sum 98 %) (EFFA, 2010a).

Table 1: Specification Summary of the Substances in the JECFA Flavouring Group of Pyrazines (JECFA, 2001c)

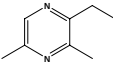
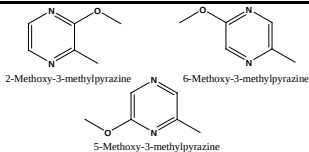
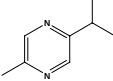
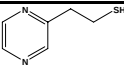
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	EFSA comments
14.022 762	Ethylpyrazine		3281 2213 13925-00-3	Liquid C ₆ H ₈ N ₂ 108.14	Soluble Miscible	152 IR 98 %	1.493-1.508 0.981-1.000	
14.024 776	2-Ethyl-3,5-dimethylpyrazine		3150 2245 13925-07-0	Liquid C ₈ H ₁₂ N ₂ 136.20	Soluble Miscible	180 IR MS 95 %	1.496-1.502 0.952-0.961	
14.025 788	2,5 or 6-Methoxy-3-methylpyrazine	 2-Methoxy-3-methylpyrazine 6-Methoxy-3-methylpyrazine 5-Methoxy-3-methylpyrazine	3183 2266 63450-30-6	Liquid C ₆ H ₈ ON ₂ 124.14	Soluble Miscible	80-85 (13 hPa) IR 97 %	1.505-1.510 1.060-1.090	Mixture of positional isomers (2/5/6-MeO-3-Me), 2-3- (75-85 %); 6-3- (15-25 %) and 5-3- isomers (1-2 %) (sum 97 %) (EFSA, 2010a).
14.026 772	2-Isopropyl-5-methylpyrazine		3554 2268 13925-05-8	Liquid C ₈ H ₁₂ N ₂ 136.20	Soluble Miscible	190 NMR 97 %	1.492-1.498 0.977-0.984	
14.027 761	2-Methylpyrazine		3309 2270 109-08-0	Liquid C ₅ H ₆ N ₂ 94.12	Soluble Miscible	137 IR 98 %	1.501-1.509 1.007-1.033	
14.028 798	5-Methylquinoxaline		3203 2271 13708-12-8	Liquid C ₉ H ₈ N ₂ 144.18	Freely soluble Very soluble	120 (20 hPa) 20 IR 98 %	1.616-1.624 1.102-1.128	
14.031 795	Pyrazineethanethiol		3230 2285 35250-53-4	Liquid C ₆ H ₈ N ₂ S 140.21	Soluble Miscible	105-110 (26hPa) IR 97 %	1.553-1.570 1.147-1.157	
14.032 784	Acetylpyrazine		3126 2286 22047-25-2	Solid C ₆ H ₆ ON ₂ 122.13	Slightly soluble Moderately soluble	188 74-80 IR 99 %	n.a. n.a.	
14.034 796	Pyrazinyl methyl sulfide		3231 2288 21948-70-9	Solid C ₅ H ₆ N ₂ S 126.18	Soluble Very soluble	75 (7 hPa) 42-47 IR 99 %	n.a. n.a.	

Table 1: Specification Summary of the Substances in the JECFA Flavouring Group of Pyrazines (JECFA, 2001c)

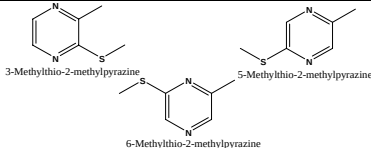
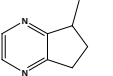
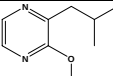
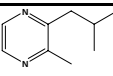
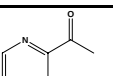
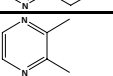
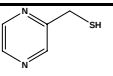
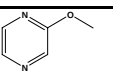
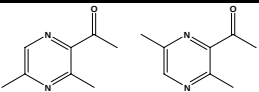
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	EFSA comments
14.035 797	2-Methyl-3,5 or 6-methylthiopyrazine		3208 2290 67952-65-2	Liquid $C_6H_8N_2S$ 140.21	Miscible Miscible	85-87 (13 hPa) IR 99 %	1.570-1.590 1.133-1.153	Mixture of positional isomers (2-Me-3/5/6-Methylthio), 2-methyl-3-methylthio-isomer (75-85 %); main SCs: 2-5-isomer (15-25 %) and 2-6-isomer (1-2 %) (EFFA, 2010a).
14.037 781	6,7-Dihydro-5-methyl-5H-cyclopentapyrazine		3306 2314 23747-48-0	Liquid $C_8H_{10}N_2$ 134.18	Slightly soluble Miscible	200 IR 97 %	1.525-1.535 1.048-1.059	Racemate (EFFA, 2010a)
14.043 792	2-Isobutyl-3-methoxypyrazine		3132 11338 24683-00-9	Liquid $C_9H_{14}ON_2$ 166.22	Soluble Miscible	60 (3 hPa) IR 95 %	1.487-1.497 0.983-1.003	
14.044 773	2-Isobutyl-3-methylpyrazine		3133 13925-06-9	Liquid $C_9H_{14}N_2$ 150.22	Soluble Miscible	199 IR 98 %	1.488-1.498 0.936-0.942	
14.049 785	2-Acetyl-3-ethylpyrazine		3250 11293 32974-92-8	Liquid $C_8H_{10}ON_2$ 150.18	Soluble Miscible	220 IR 98 %	1.509-1.520 1.068-1.079	
14.050 765	2,3-Dimethylpyrazine		3271 11323 5910-89-4	Liquid $C_6H_8N_2$ 108.14	Soluble Miscible	156 IR 95 %	1.501-1.510 0.997-1.030	Mixture of positional isomers (2,3-; 2,5- and 2,6-isomers), 2,3- (70-85 %); 2,5- (10-25 %) and 2,6-isomers (1-2 %) (sum 95 %) (EFFA, 2010a).
14.053 794	Mercaptomethylpyrazine		3299 11502 59021-02-2	Liquid $C_5H_6N_2S$ 126.18	Soluble Miscible	94 (13 hPa) IR 98 %	1.548-1.560 1.148-1.156	
14.054 787	Methoxypyrazine		3302 11347 3149-28-8	Liquid $C_5H_6ON_2$ 110.12	Miscible Miscible	61-62 (38 hPa) IR 99 %	1.492-1.510 1.110-1.140	Specific gravity: depending on the quality and the producer, the SG ranges from: 1.110-1.140 (EFFA, 2010a).
14.055 786	2-Acetyl-3,5-dimethylpyrazine		3327 11294 54300-08-2	Liquid $C_8H_{10}ON_2$ 150.18	Soluble Miscible	70 (9 hPa) IR 97 %	1.510-1.520 1.070-1.075	Mixture of positional isomers (2-Acetyl-3,5/6-dimethyl), 3,5-dimethyl (65-70 %) and 3,6-dimethyl (25-30 %) isomers (sum 95 %) (EFFA, 2010a).

Table 1: Specification Summary of the Substances in the JECFA Flavouring Group of Pyrazines (JECFA, 2001c)

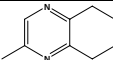
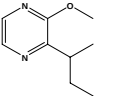
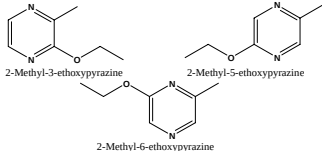
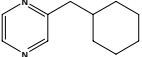
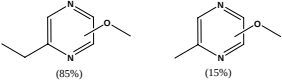
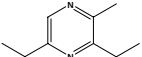
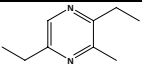
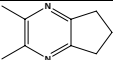
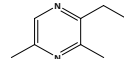
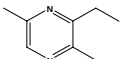
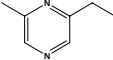
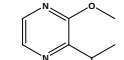
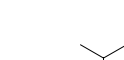
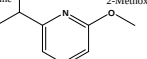
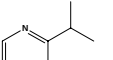
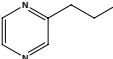
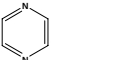
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	EFSA comments
14.056 777	2,3-Diethyl-5-methylpyrazine		3336 11303 18138-04-0	Liquid C ₉ H ₁₄ N ₂ 150.22	Moderately soluble Miscible	203 IR 98 %	1.493-1.505 0.938-0.957	
14.062 791	2-(sec-Butyl)-3-methoxypyrazine		3433 11300 24168-70-5	Liquid C ₉ H ₁₄ ON ₂ 166.22	Soluble Miscible	50 (1 hPa) IR 99 %	1.478-1.498 0.976-1.002	Racemate (EFFA, 2010a). Specific gravity: depending on the quality and the producer, the SG ranges from: 0.976-1.002 (EFFA, 2010a).
14.067 793	2-Methyl-3,5 or 6-ethoxypyrazine		3569 11921 32737-14-7	Liquid C ₇ H ₁₀ ON ₂ 138.17	Soluble Miscible	175-176 IR 97 %	1.493-1.497 1.034-1.041	Mixture of positional isomers (2-Methyl-3/5/6- ethoxy-), 2-methyl-3- ethoxypyrazine (75-85 %), 2-methyl-5-ethoxypyrazine (15-25 %) and 2-methyl-6- ethoxypyrazine (1-2 %) (sum 97 %) (EFFA, 2010a).
14.069 783	Cyclohexylmethylpyrazine		3631 28217-92-7	Liquid C ₁₁ H ₁₆ N ₂ 176.26	Slightly soluble Miscible	100 (5 hPa) NMR 97 %	1.515-1.520 1.003-1.009	
14.077 789	2-Ethyl-(3,5 or 6)-methoxypyrazine (85%) and 2-Methyl-(3,5 or 6)-methoxypyrazine (13%)		3280 11329	Liquid	Soluble Miscible	80-95 (13 hPa) IR 99 %	1.497-1.505 1.036-1.052	Mixture of positional isomers (2-Ethyl/Methyl- (3/5/6)-methoxypyrazine: 2- Ethyl form (85 %) & 2- Methyl form (13 %), 2-3- (75-85 %); 2-5- (15-25 %) and 2-6- ethyl or methyl (1- 2 %) (EFFA, 2010a).
14.082 950	2-Acetyl-3-methylpyrazine		3964 11296 23787-80-6	Liquid C ₇ H ₈ ON ₂ 136.15	Soluble Miscible	90 (26 hPa) IR 98 %	1.521-1.523 1.105-1.114	
14.095 779	3,5-Diethyl-2-methylpyrazine		3916 11305 18138-05-1	Liquid C ₉ H ₁₄ N ₂ 150.22	Slightly soluble Miscible	95 (18 hPa) NMR 97 %	1.492-1.502 0.944-0.954	
14.096 778	2,5-Diethyl-3-methylpyrazine		3915 11304 32736-91-7	Liquid C ₉ H ₁₄ N ₂ 150.22	Moderately soluble Miscible	95 (18 hPa) NMR 97 %	1.4922- 1.5022 0.944-0.954	

Table 1: Specification Summary of the Substances in the JECFA Flavouring Group of Pyrazines (JECFA, 2001c)

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)	EFSA comments
14.098 782	6,7-Dihydro-2,3-dimethyl-5H-cyclopentapyrazine		3917 11309 38917-62-3	Solid $C_9H_{12}N_2$ 148.21	Slightly soluble Very soluble	66 (3 hPa) 25-27 NMR 97 %	n.a. n.a.	CASrn in Register refers to 6,7-dihydro-3,5-dimethyl-5H-cyclopentapyrazine. CASrn to be changed.
14.100 775	3,(5- or 6-)-Dimethyl-2-ethylpyrazine	  2-Ethyl-3,5-dimethylpyrazine 2-Ethyl-3,6-dimethylpyrazine	3149 727 55031-15-7	Liquid $C_8H_{12}N_2$ 136.2	Soluble Miscible	180 IR NMR 95 %	1.496-1.506 0.950-0.980	Mixture of positional isomers (2-Ethyl-3,5/6-dimethyl-), 2-ethyl-3,5-dimethylpyrazine (50-57 %); 2-ethyl-3,6-dimethylpyrazine (43-50 %) (EFFA, 2010a).
14.114 769	2-Ethyl-6-methylpyrazine		3919 11331 13925-03-6	Liquid $C_7H_{10}N_2$ 122.17	Soluble Miscible	80 (65 hPa) IR 95 %	1.487-1.497 0.967-0.980	Mixture of positional isomers (2-Ethyl-5/6-ethylpyrazine), 2-5- (66 %) and 2-6-isomers (33%) (sum 97 %) (EFFA, 2010a).
14.121 790	2-Isopropyl-(3,5 or 6)-methoxypyrazine	  2-Methoxy-3-isopropylpyrazine 2-Methoxy-5-isopropylpyrazine  2-Methoxy-6-isopropylpyrazine	3358 11344 93905-03-4	Liquid $C_8H_{12}ON_2$ 152.2	Soluble Miscible	120-125 (26hPa) IR 97 %	1.492-1.499 1.010-1.022	Mixture of positional isomers (2-Isopropyl-3/5/6-methoxy), 2-3- (75-85 %); 2-5- (15-25 %) and 2-6-isomers (1-2 %) (sum 97 %) (EFFA, 2010a).
14.123 764	Isopropylpyrazine		3940 11343 29460-90-0	Liquid $C_7H_{10}N_2$ 122.17	Soluble Miscible	70 (26 hPa) IR 98 %	1.486-1.496 0.967-0.972	
14.142 763	Propylpyrazine		3961 11362 18138-03-9	Liquid $C_7H_{10}N_2$ 122.17	Soluble Miscible	65 (16 hPa) NMR 98 %	1.492-1.496 0.966-0.970	
14.144 951	Pyrazine		4015 11363 290-37-9	Solid $C_4H_4N_2$ 80.09	Freely soluble Very soluble	115-118 53 IR 98 %	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.

TABLE 2: GENOTOXICITY DATA

Table 2.1: Genotoxicity Data (in vitro / in vivo) for 41 Pyrazines (JECFA, 2002a)

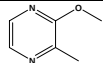
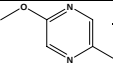
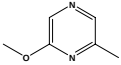
Table 2.1: Summary of Genotoxicity Data for 41 Pyrazines (JECFA, 2002a)

FL-no JECFA-no	EU Register name JECFA name	Structural formula	End-point	Test system	Concentration *)	Results	Reference
In vitro							
14.019 774	2,3,5-Trimethylpyrazine		Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA102	0.98–97 735 mg/plate	Negative ^a	(Aeschbacher et al., 1989)
14.020 766	2,5-Dimethylpyrazine		Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Not reported	Negative ^a	(Lee et al., 1994a)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1537	12 500–200 000 mg/plate	Negative ^a	(Stich et al., 1980)
			Mutation	<i>S. cerevisiae</i> D5	16 900–135 000 mg/ml	Positive ^b	(Stich et al., 1980)
			Chromosomal aberration	Chinese hamster ovary cells	2500–40 000 mg/ml	Positive ^a	(Stich et al., 1980)
14.021 767	2,6-Dimethylpyrazine		Reverse mutation	<i>S. typhimurium</i> TA100 <i>S. typhimurium</i> TA 98 <i>S. typhimurium</i> TA 98	86–10 800 mg/plate 2160–10 800 mg/plate 86–10 800 mg/plate	Negative ^a Positive ^b Negative ^c	(Lee et al., 1994a)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA102	0.54–54 000 mg/plate	Negative ^a	(Aeschbacher et al., 1989)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1537	6300–100 000 mg/plate	Negative ^a	(Stich et al., 1980)
			Mutation	<i>S. cerevisiae</i> D5	3300–33 800 mg/ml	Positive ^b	(Stich et al., 1980)
			Chromosomal aberration	Chinese hamster ovary cells	5000–10 000 mg/ml	Positive ^a	(Stich et al., 1980)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA102	0.97–97 200 mg/plate	Negative ^a	(Aeschbacher et al., 1989)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1537	6300–100 000 mg/plate	Negative ^a	(Stich et al., 1980)
14.022 762	Ethylpyrazine 2-Ethylpyrazine		Mutation	<i>S. cerevisiae</i> D5	8500–67 500 mg/ml	Positive ^b	(Stich et al., 1980)
			Chromosomal aberration	Chinese hamster ovary cells	2500–5000 mg/ml	Positive ^a	(Stich et al., 1980)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA102	0.97–97 200 mg/plate	Negative ^a	(Aeschbacher et al., 1989)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1537	6300–100 000 mg/plate	Negative ^a	(Stich et al., 1980)
14.024 776	2-Ethyl-3,5-dimethylpyrazine 3-Ethyl-2,6-dimethylpyrazine		Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA102	0.97–97 200 mg/plate	Negative ^a	(Aeschbacher et al., 1989)
14.025 788	2,5 or 6-Methoxy-3-methylpyrazine (2 or 5 or 6)-Methoxy-3-methylpyrazine		Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538	3600 mg/plate	Negative ^a	(Wild et al., 1983)
							
							
14.027 761	2-Methylpyrazine		Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA102	0.94–94,000 mg/plate	Negative ^a	(Aeschbacher et al., 1989)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Not reported	Negative ^a	(Lee et al., 1994a)
			Reverse mutation	<i>S. typhimurium</i> TA 98, TA100, TA1537	6300–100 000 mg/plate	Negative ^a	(Stich et al., 1980)

Table 2.1: Summary of Genotoxicity Data for 41 Pyrazines (JECFA, 2002a)

FL-no JECFA-no	EU Register name JECFA name	Structural formula	End-point	Test system	Concentration *)	Results	Reference
14.050 765	2,3-Dimethylpyrazine		Mutation	<i>S. cerevisiae</i> D5	8500–67 500 mg/ml	Positive ^b	(Stich et al., 1980)
			Chromosomal aberration	Chinese hamster ovary cells	2500–40 000 mg/ml	Positive ^a	(Stich et al., 1980)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA102	0.97–97 200 mg/plate	Negative ^a	(Aeschbacher et al., 1989)
14.144 951	Pyrazine		Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Not reported	Negative ^a	(Lee et al., 1994a)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA102	0.64–64 000 mg/plate	Negative ^a	(Aeschbacher et al., 1989)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100	Not reported	Negative ^a	(Lee et al., 1994a)
			Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1537	6300–100 000 mg/plate	Negative ^a	(Stich et al., 1980)
			Mutation	<i>S. cerevisiae</i> D5	7500–60 000 mg/ml	Positive ^b	(Stich et al., 1980)
			Chromosomal aberration	Chinese hamster ovary cells	2500–25 000 mg/ml	Positive ^a	(Stich et al., 1980)

In vivo

14.025 788	2,5 or 6-Methoxy-3-methylpyrazine (2 or 5 or 6)-Methoxy-3-methylpyrazine	  2-Methoxy-3-methylpyrazine 6-Methoxy-3-methylpyra  5-Methoxy-3-methylpyrazine	Basic mutation	<i>Drosophila melanogaster</i>	10 mmol/L	Negative	(Wild et al., 1983)
			Micronucleus formation	Mouse	87, 174, or 248 mg/kg bw	Negative	(Wild et al., 1983)

*: Concentration should be in microgram not mg..A mistake in the JECFA monograph.

a: With and without metabolic activation.

b: Without metabolic activation.

c: With metabolic activation.

Table 2.2: Genotoxicity Data (in vitro) EFSA / FGE.17Rev2

Substances listed in brackets are the JECFA evaluated supporting substances in FGE.17Rev2

Table 2.2: Summary of Genotoxicity Data (in vitro) EFSA, FGE.17Rev2

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
(Pyrazine [14.144])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	64000 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	64000 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Ames test	<i>S. typhimurium</i> TA98; TA100	Not reported	Negative ¹	(Lee et al., 1994a)	Report of insufficient quality because test concentrations are not given. Reference compound within a large study, details are reported for positive compounds only.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1537	100000 µg/plate	Negative ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1535; TA1537; TA1538	10000 µg/ml	Negative ^{1,2}	(Fung et al., 1988)	Valid study in accordance with OECD guideline 471.
	Mutation assay	<i>S. cerevisiae</i> Strain D5	60000 µg/ml	Positive ³	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Chromosomal Aberration assay	Chinese hamster ovary cells	10000 µg/ml 2500 µg/ml	Positive ¹ Positive ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
(2-Methylpyrazine [14.027])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	94000 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	94000 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Ames test	<i>S. typhimurium</i> TA98; TA100	Not reported	Negative ¹	(Lee et al., 1994a)	Report of insufficient quality because test concentrations are not given. Reference compound of a large study, details are reported for positive compounds only.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1537	100000 µg/plate	Negative ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Mutation assay	<i>S. cerevisiae</i> Strain D5	67500 µg/ml	Positive ³	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Chromosomal Aberration assay	Chinese hamster ovary cells	40000 µg/ml 20000 µg/ml	Positive ¹ Positive ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	97200 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	97200 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
(Ethylpyrazine [14.022])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1537	100000 µg/plate	Negative ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Mutation assay	<i>S. cerevisiae</i> Strain D5	67500 µg/ml	Positive ³	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Chromosomal Aberration assay	Chinese hamster ovary cells	5000 µg/ml 2500 µg/ml	Positive ¹ Positive ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	97200 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	97200 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
(2,3-Dimethylpyrazine [14.050])	Ames test	<i>S. typhimurium</i> TA98; TA100	NR	Negative ¹	(Lee et al., 1994a)	Report of insufficient quality because test concentrations are not given. Reference compound within a large study, details are reported for positive compounds only.

Table 2.2: Summary of Genotoxicity Data (*in vitro*) EFSA, FGE.17Rev2

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
(2,5-Dimethylpyrazine [14.020])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	97200 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	97200 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Ames test	<i>S. typhimurium</i> TA98; TA100	Not reported	Negative ¹	(Lee et al., 1994a)	Report of insufficient quality because test concentrations are not given. Reference compound of a large study, details are reported for positive compounds only.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1537	200000 µg/plate	Negative ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Mutation assay	<i>S. cerevisiae</i> Strain D5	135500 µg/ml	Positive ³	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Chromosomal aberration assay	Chinese hamster ovary cells	40000 µg/ml 20000 µg/ml	Positive ¹ Positive ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
(2,6-Dimethylpyrazine [14.021])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	54000 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	54000 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Ames test	<i>S. typhimurium</i> TA98; TA100	10800	Negative ⁴	(Lee et al., 1994a)	Well conducted study, valid although not in accordance with OECD guideline 471: two <i>S. typhimurium</i> strains only.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1537	100000 µg/plate	Negative ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Mutation assay	<i>S. cerevisiae</i> Strain D5	33800 µg/ml	Positive ³	(Stich et al., 1980)	Study with strong limitations with results of limited value.
	Chromosomal aberration assay	Chinese hamster ovary cells	10000 µg/ml 2500 µg/ml	Positive ¹ Positive ¹	(Stich et al., 1980)	Study with strong limitations with results of limited value.
(2,3-Diethylpyrazine [14.005])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	109000 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	109000 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
(2,3,5-Trimethylpyrazine [14.019])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	97735 µg/plate	Negative ¹	(Aeschbacher et al., 1989)	97735 µg/plate: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only.
((2,5 or 6)-Methoxy-3-methylpyrazine [14.025])	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1535; TA1537; TA1538	3600 µg/plate	Negative ¹	(Wild et al., 1983)	Well conducted study, valid although not in accordance with OECD guideline 471: test concentrations not reported.
(Pyrazinylethanethiol [14.031])	Ames test	<i>S. typhimurium</i> TA97; TA98; TA100; TA1535	NR	Negative ¹	(Zeiger and Margolin, 2000)	Well conducted study, valid although not in accordance with OECD guideline 471: report does not give test concentrations, four test concentrations.
Quinoxaline [14.147]	Ames test	<i>S. typhimurium</i> TA98; TA100	NR	Negative ³	(Beutin et al., 1981)	TA98 ; TA100: results presented in detail, without metabolic activation. TA1535,TA1537,TA1538: results incl. metabolic activation are mentioned in text (negative), but no data given. Not in accordance with OECD guideline 471.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	0.35 mmol	Negative ^{3,5}	(Aeschbacher et al., 1989)	0.35 mmol: highest non-bactericidal dose. Well conducted study, valid although not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
	Modified Ames test	<i>S. typhimurium</i> TA98; TA100; TA1535; TA1537; TA1538; G46; C3076; D3052 <i>E. coli</i> WP2; WP2uvrA	NR	Negative ³	(McMahon et al., 1979)	Review, of limited value (concentrations tested not reported).
	Ames test (plate incorporation method)	<i>S. typhimurium</i> TA98; TA100; TA1535; TA1537; TA1538	10000 µg/plate	Negative ³	(San, 1995)	Valid study in accordance with OECD guideline 471.

Table 2.2: Summary of Genotoxicity Data (*in vitro*) EFSA, FGE.17Rev2

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
	DNA Polymerase deficiency assay	<i>E. coli</i>	NR	Negative ³	(Rosenkranz & Leifer, 1980)	Review, of limited value (concentrations tested not reported; without metabolic activation).
	SOS Chromosome test	<i>E. coli</i> PQ37	NR	Negative ¹	(Beutin et al., 1981)	
	Mouse lymphoma mutagenesis assay	L5178Y TK ^{+/+} mouse lymphocytes	(with S9) 20 – 250 (without S9) 100 – 1500 microg/ml	Positive ⁶ Weakly Positive ³	(National Cancer Institute, 1998)	Valid study in accordance with OECD guideline 476.
2-Methylquinoxaline [14.139]	Ames test	<i>S. typhimurium</i> TA98; TA100	500 µg/plate	Positive ¹	(Hashimoto et al., 1979)	Well conducted study, valid although not in accordance with OECD guideline 471: two <i>S. typhimurium</i> strains only, highest dose but not individual doses reported. Positive only in TA98 and T100 with metabolic activation.
	Ames test	<i>S. typhimurium</i> TA98; TA100; TA102	0.007 - 700 µmol/plate (equal to 0.001 – 100 mg/plate)	Negative ^{1, 7}	(Aeschbacher et al., 1989)	0.7 mmol: highest non-bactericidal dose. Well conducted study (valid), but not in accordance with OECD guideline 471: three <i>S. typhimurium</i> strains only, dose range but not individual doses reported.
2,3-Dimethylquinoxaline [14.108]	Ames test	<i>S. typhimurium</i> TA98; TA100; TA1535;	2500 µg/plate	Negative ⁶	(Anderson and Styles, 1978)	Well conducted study, valid although not in accordance with OECD guideline 471 (with S9 metabolic activation only).
	Ames test	<i>S. typhimurium</i> TA100	NR	Negative ⁶	(Epler et al., 1978)	Review, no detailed information on test conditions incl. concentration. Authors pointed out the unanswered question whether the testing of negative compounds can sensibly be terminated (in 1978).
	Ames test	<i>S. typhimurium</i> TA98; TA100	NR	Negative ¹	(Hashimoto et al., 1979)	Validity cannot be evaluated. Concentrations not reported. Results not reported in detail.
(5-Methylquinoxaline [14.028])	Reverse mutation	<i>S. typhimurium</i> TA 98, TA100, TA1535, TA1537 and <i>E. coli</i> strain WP2 uvrA	Up to 5000 microgram/plate	Negative ¹	(Ogura & Wakamatsu, 2004)	Valid. GLP-study in compliance with OECD 471 (except that no justification was provided for the use of duplicate instead of triplicate plating).
	Chromosomal aberration assay	Chinese hamster lung-derived CHL/IU cells	320, 480, 720 microgram/ml 72, 228, 720 microgram/ml	Negative ³ Positive ⁶	(Ajimu & Kawaguchi, 2004a)	Valid. GLP-study mainly in compliance with OECD 473 (duration of exposure not clearly reported). The authors noted in the discussion section that cytotoxicity was observed in the form of decreased cell viability and reproductive rate. However, it is not clear if one or two parameters for cytotoxicity were measured. The percentage of “cell productivity” (the cell number was measured and expressed as relative growth rate compared to negative control) was reported. According to the authors, there was a clear evidence of cytotoxicity in the form of decreased cell viability and reproductive rate at concentrations where chromosomal aberrations were observed. However, the results presented in tables demonstrate that 30 and 66 % of cell with chromosomal aberrations were induced at the limit of excessive cytotoxicity (54 and 46% of relative growth) in the preliminary test (in which 50 cells per slide were scored) at 180 and 360 µg/mL in the presence of S9, respectively. In the main test, the percentage of cells with chromosomal aberrations in the presence of S9 was 2.0, 2.5, 6.5 and 57.5 at 0, 72, 228 and 720 µg/mL, respectively, which was accompanied by 100, 90, 85 and 46% relative growth, respectively.

NR: Not reported.

¹ With and without S9 metabolic activation.

² Metabolic activation was provided with both rat and hamster liver S9 mix.

³ Without S9 metabolic activation.

- ⁴ Results were negative in TA100 with and without S9 metabolic activation; however, in TA98 the results were negative and positive with and without S9 metabolic activation, respectively.
- ⁵ Results were negative in TA100 with and without S9 metabolic activation. Weak results were noted in TA98 and TA102 with S9 metabolic activation. These changes may be related to the heat production products of the Maillard reaction in the presence of creatinine.
- ⁶ With S9 metabolic activation.
- ⁷ Weak results were noted in all strains with S9 metabolic activation. (*the number of revertants was increased up to 1.3-fold compared to control*). According to the authors (Aeschbacher et al., 1989), these changes may be related to the heat production products of the Maillard reaction in the presence of creatinine.

Table 2.3: Genotoxicity Data (in vivo) EFSA / FGE.17Rev2

Substances listed in brackets are JECFA-evaluated supporting substances in FGE.17Rev2.

Table 2.3: GENOTOXICITY (in vivo)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments
(2,5 or 6)-Methoxy-3- methylpyrazine [14.025])	Basic test	<i>D. melanogaster</i>		10 mM	Negative	(Wild et al., 1983)	Limited relevance for risk assessment as the test is not in a mammalian system and the test is not used routinely.
	Micronucleus assay	Mouse		248 mg/kg	Negative	(Wild et al., 1983)	Study design does not meet the criteria of current guidelines (PCE/NCE ratio was not reported, thus it is not clear if the test substance reached the bone marrow). Not in accordance with OECD guideline 474 (1983/1997).
Quinoxaline [14.147]	Sperm head abnormality test	Mouse	I.P	2500 mg/kg	Negative	(Topham, 1980)	Sperm head abnormality test does not make use of a genetic endpoint.
(5-Methylquinoxaline [14.028])	Micronucleus assay	Mouse	Gavage	125, 250 and 500 mg/kg/day	Negative	(Ajimu & Kawaguchi, 2004b)	Valid. GLP-study mainly in compliance with OECD 474 (only 5 male mice per group instead of 5 males and 5 females).

TABLE 3: SUMMARY OF SAFETY EVALUATIONS

Table 3.1: Summary of Safety Evaluation of Pyrazine and 40 Derivatives (JECFA, 2003a)

Table 3.1: Summary of Safety Evaluation of JECFA-Evaluated Pyrazine Derivatives (JECFA, 2003a)

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI 1) US MSDI (µg/capita/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	EFSA conclusion on the named compound (Procedure steps, intake estimates, NOEL, genotoxicity)	EFSA conclusion on the material of commerce
14.005 771	2,3-Diethylpyrazine		1.6 1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.006 768	2-Ethyl-3-methylpyrazine		72 9	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.017 770	2-Ethyl-5-methylpyrazine		4.0 1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.018 780	2,3,5,6-Tetramethylpyrazine		6.7 19	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.019 774	2,3,5-Trimethylpyrazine		100 46	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.020 766	2,5-Dimethylpyrazine		19 8	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.021 767	2,6-Dimethylpyrazine		1.3 2	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.022 762	Ethylpyrazine		2.2 6	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.024 776	2-Ethyl-3,5-dimethylpyrazine		1.2 0.3	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.

Table 3.1: Summary of Safety Evaluation of JECFA-Evaluated Pyrazine Derivatives (JECFA, 2003a)

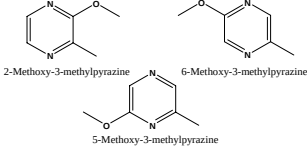
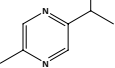
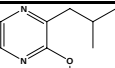
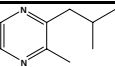
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI 1) US MSDI (µg/capita/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	EFSA conclusion on the named compound (Procedure steps, intake estimates, NOAEL, genotoxicity)	EFSA conclusion on the material of commerce
14.025 788	2,5 or 6-Methoxy-3-methylpyrazine	 2-Methoxy-3-methylpyrazine 6-Methoxy-3-methylpyrazine 5-Methoxy-3-methylpyrazine	2.2 15	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.026 772	2-Isopropyl-5-methylpyrazine		0.024 0.4	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.027 761	2-Methylpyrazine		17 7	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.032 784	Acetylpyrazine		12 120	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.037 781	6,7-Dihydro-5-methyl-5H-cyclopentapyrazine		3.9 4	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.043 792	2-Isobutyl-3-methoxypyrazine		1.6 1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.044 773	2-Isobutyl-3-methylpyrazine		0.037 0.01	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.049 785	2-Acetyl-3-ethylpyrazine		0.73 0.1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.050 765	2,3-Dimethylpyrazine		14 4	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.054 787	Methoxypyrazine		3.0 1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.

Table 3.1: Summary of Safety Evaluation of JECFA-Evaluated Pyrazine Derivatives (JECFA, 2003a)

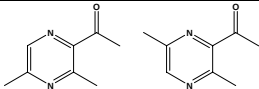
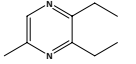
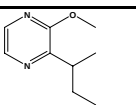
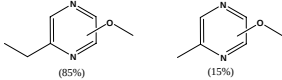
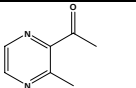
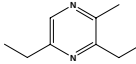
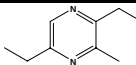
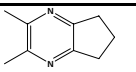
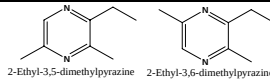
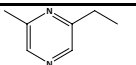
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI 1) US MSDI ($\mu\text{g}/\text{capita}/\text{day}$)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	EFSA conclusion on the named compound (Procedure steps, intake estimates, NOAEL, genotoxicity)	EFSA conclusion on the material of commerce
14.055 786	2-Acetyl-3,5-dimethylpyrazine		0.97 1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.056 777	2,3-Diethyl-5-methylpyrazine		0.11 1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.062 791	2-(sec-Butyl)-3-methoxypyrazine		0.85 0.1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.077 789	2-Ethyl-(3,5 or 6)-methoxypyrazine (85%) and 2-Methyl-(3,5 or 6)-methoxypyrazine (13%)		1.3 1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.082 950	2-Acetyl-3-methylpyrazine		0.1 0.1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.095 779	3,5-Diethyl-2-methylpyrazine		0.012 0.01	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.096 778	2,5-Diethyl-3-methylpyrazine		0.012 0.01	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.098 782	6,7-Dihydro-2,3-dimethyl-5H-cyclopentapyrazine		0.012 0.01	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach. CASrn in Register refers to 6,7-dihydro-3,5-dimethyl-5H-cyclopentapyrazine. CASrn to be changed.
14.100 775	3,(5- or 6-)-Dimethyl-2-ethylpyrazine		38 9	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.114 769	2-Ethyl-6-methylpyrazine		0.37 0.4	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI	No safety concern at the estimated level of intake based on the MSDI

Table 3.1: Summary of Safety Evaluation of JECFA-Evaluated Pyrazine Derivatives (JECFA, 2003a)

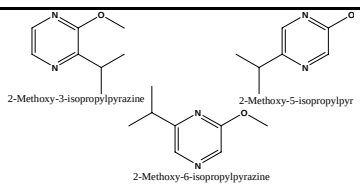
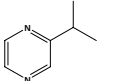
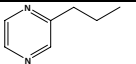
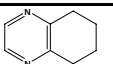
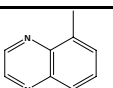
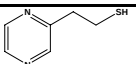
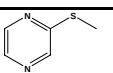
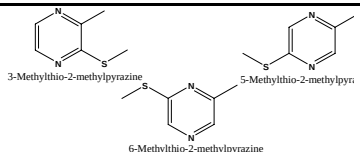
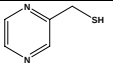
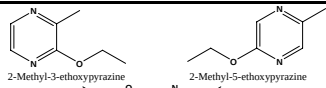
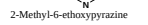
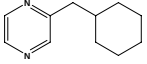
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI 1) US MSDI ($\mu\text{g/capita/day}$)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	EFSA conclusion on the named compound (Procedure steps, intake estimates, NOAEL, genotoxicity)	EFSA conclusion on the material of commerce
14.121 790	2-Isopropyl-(3,5 or 6)- methoxypyrazine	 2-Methoxy-3-isopropylpyrazine 2-Methoxy-5-isopropylpyrazine 2-Methoxy-6-isopropylpyrazine	0.0012 0.1	Class II A3: Intake below threshold	4)	approach No safety concern at the estimated level of intake based on the MSDI approach	approach. No safety concern at the estimated level of intake based on the MSDI approach.
14.123 764	Isopropylpyrazine		0.12 0.1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.142 763	Propylpyrazine		0.12 0.1	Class II A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.015 952	5,6,7,8-Tetrahydroquinoxaline		8 ND	Class III A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.028 798	5-Methylquinoxaline		22 1	Class III A3: Intake below threshold	4)	The Panel concluded based on additional data that the genotoxicity alert can be ruled out. No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.031 795	Pyrazineethanethiol		0.13 1	Class III A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.034 796	Pyrazinyl methyl sulfide		0.0061 0.01	Class III A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.035 797	2-Methyl-3,5 or 6- methylthiopyrazine	 3-Methylthio-2-methylpyrazine 5-Methylthio-2-methylpyrazine 6-Methylthio-2-methylpyrazine	6.3 13	Class III A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.

Table 3.1: Summary of Safety Evaluation of JECFA-Evaluated Pyrazine Derivatives (JECFA, 2003a)

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI 1) US MSDI (µg/capita/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	EFSA conclusion on the named compound (Procedure steps, intake estimates, NOAEL, genotoxicity)	EFSA conclusion on the material of commerce
14.053 794	Mercaptomethylpyrazine		0.012 0.01	Class III A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.067 793	2-Methyl-3,5 or 6-ethoxypyrazine	 	0.055 0.01	Class III A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.069 783	Cyclohexylmethylpyrazine		0.012 0.01	Class III A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.
14.144 951	Pyrazine		0.024 0.2	Class III A3: Intake below threshold	4)	No safety concern at the estimated level of intake based on the MSDI approach	No safety concern at the estimated level of intake based on the MSDI approach.

- 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 µg/person/day Class II = 540 µg/person/day, 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.

ND: not determined.

Table 3.2: Summary of Safety Evaluation Applying the Procedure (EFSA / FGE.17Rev2)

Table 3.2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

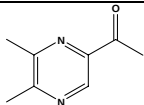
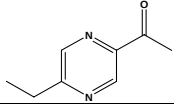
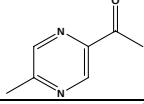
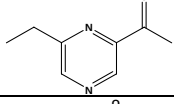
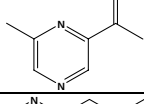
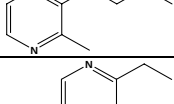
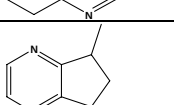
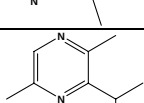
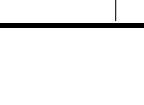
FL-no	EU Register name	Structural formula	MSDI 1) (µg/capita/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
14.081	5-Acetyl-2,3-dimethylpyrazine		0.012	Class II A3: Intake below threshold	4)	6)	
14.083	2-Acetyl-5-ethylpyrazine		0.012	Class II A3: Intake below threshold	4)	6)	
14.084	2-Acetyl-5-methylpyrazine		0.0024	Class II A3: Intake below threshold	4)	6)	
14.086	2-Acetyl-6-ethylpyrazine		0.0061	Class II A3: Intake below threshold	4)	6)	
14.087	2-Acetyl-6-methylpyrazine		0.028	Class II A3: Intake below threshold	4)	6)	
14.091	2-Butyl-3-methylpyrazine		0.12	Class II A3: Intake below threshold	4)	6)	
14.097	2,5-Diethylpyrazine		0.024	Class II A3: Intake below threshold	4)	6)	
14.099	6,7-Dihydro-5,7-dimethyl-5H-cyclopentapyrazine		0.032	Class II A3: Intake below threshold	4)	7)	
14.101	2,5-Dimethyl-3-isopropylpyrazine		0.018	Class II A3: Intake below threshold	4)	6)	

Table 3.2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

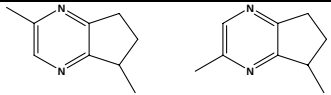
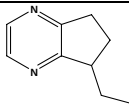
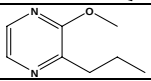
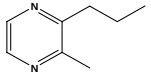
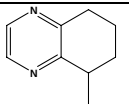
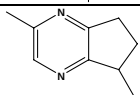
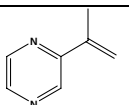
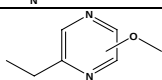
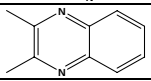
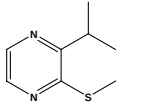
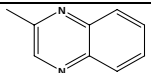
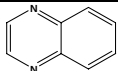
FL-no	EU Register name	Structural formula	MSDI 1) ($\mu\text{g/capita/day}$)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
14.102	5,6-Dimethyldihydrocyclopentapyrazine		0.024	Class II A3: Intake below threshold	4)	7)	
14.113	5-Ethyl-6,7-dihydro-5H-cyclopentapyrazine		0.012	Class II A3: Intake below threshold	4)	6)	
14.127	2-Methoxy-3-propylpyrazine		0.061	Class II A3: Intake below threshold	4)	6)	
14.129	2-Methyl-3-propylpyrazine		0.011	Class II A3: Intake below threshold	4)	6)	
14.148	5,6,7,8-Tetrahydro-5-methylquinoxaline		0.0073	Class II A3: Intake below threshold	4)	6)	
14.161	6,7-Dihydro-2,5-dimethyl-5H-cyclopentapyrazine		0.011	Class II A3: Intake below threshold	4)	6)	
14.052	Isopropenylpyrazine		0.012	Class II B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
14.051	2,5 or 6-Methoxy-3-ethylpyrazine			Class II No evaluation			
14.108	2,3-Dimethylquinoxaline		0.049	Class III A3: Intake below threshold	4)	6)	
14.122	2-Isopropyl-3-methylthiopyrazine		0.061	Class III B3: Intake below threshold, B4: Adequate NOAEL exists	4)	6)	
14.139	2-Methylquinoxaline		0.12	Class III A3: Intake below threshold	4)	6)	

Table 3.2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

FL-no	EU Register name	Structural formula	MSDI 1) (µg/capita/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
14.147	Quinoxaline		0.12	Class III No evaluation			a)

- 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 µg/person/day, Class II = 540 µg/person/day, 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.
- 6) No safety concern at estimated level of intake of the material of commerce meeting the specification of Table 1 (based on intake calculated by the MSDI approach).
- 7) Tentatively regarded as presenting no safety concern (based on intake calculated by the MSDI approach) pending further information on the purity of the material of commerce and/or information on stereoisomerism.
- 8) No conclusion can be drawn due to lack of information on the purity of the material of commerce.

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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)
CoE	Council of Europe
DNA	Deoxyribonucleic acid
EFSA	The European Food Safety Authority
EPA	United States Environmental Protection Agency
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good laboratory practise
ID	Identity
Ip	Intraperitoneal
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MNPCE	Micronucleated polychromatic erythrocytes
MSDI	Maximised Survey-derived Daily Intake
mTAMDI	Modified Theoretical Added Maximum Daily Intake
NCE	Normochromatic erythrocyte
No	Number
NOAEL	No observed adverse effect level
NTP	National Toxicology Program
PCE	Polychromatic erythrocyte
SCE	Sister chromatic exchange
SCF	Scientific Committee on Food

WHO World Health Organisation

EVALUATION OF CERTAIN FOOD ADDITIVES AND CONTAMINANTS

Fifty-seventh report of the
Joint FAO/WHO Expert Committee on
Food Additives



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Rome, 5–14 June 2001

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Monographs containing summaries of relevant data and toxicological evaluations are available from WHO under the title:

Safety evaluation of certain food additives and contaminants. WHO Food Additives Series, No. 48, 2002.

Specifications are issued separately by FAO under the title:

Compendium of food additive specifications, addendum 9. FAO Food and Nutrition Paper, No. 52, Add. 9, 2001.

INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY

The preparatory work for toxicological evaluations of food additives and contaminants by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) is actively supported by certain of the Member States that contribute to the work of the International Programme on Chemical Safety (IPCS).

The International Programme on Chemical Safety (IPCS) is a joint venture of the United Nations Environment Programme, the International Labour Organization and the World Health Organization. One of the main objectives of the IPCS is to carry out and disseminate evaluations of the effects of chemicals on human health and the quality of the environment.

1. Introduction

The Joint FAO/WHO Expert Committee on Food Additives met in Rome from 5 to 14 June 2001. The meeting was opened by Mr W. Clay, Chief, Nutrition Programmes Service, Food and Nutrition Division, FAO, on behalf of the Directors-General of the Food and Agriculture Organization of the United Nations and the World Health Organization. Mr Clay reminded the Committee that one of its tasks was to provide scientific advice to Member States of the two organizations with respect to food regulations and control. He noted that dioxins and some related compounds were to be discussed by the Committee for the first time, almost 25 years after the accident in Seveso, Italy, in which large quantities of dioxins had been released into the environment. That event had raised awareness and concern both in the general population and among regulators, leading to a greater demand for global assessment, management and communication of risks relating to environmental contamination and food. The Committee's deliberations on the topic would therefore be important and should help to improve communication between those responsible for risk assessment and risk management. Mr Clay informed the Committee that its activities would be part of a wider effort by FAO and WHO to improve food safety. The two organizations were planning to establish a Global Forum for Food Safety Regulators, in order to promote the exchange of information about ways of dealing with issues of potential importance to public health and international food trade among those responsible for regulating food safety.

2. General considerations

As a result of the recommendations of the first Joint FAO/WHO Conference on Food Additives, held in September 1955 (1), there have been fifty-six previous meetings of the Committee (Annex 1). The present meeting was convened on the basis of a recommendation made at the fifty-fifth meeting (Annex 1, reference 149).

The tasks before the Committee were:

- to elaborate further principles for evaluating the safety of food additives and contaminants (section 2);
- to undertake toxicological evaluations of certain food additives, flavouring agents and contaminants (sections 3–5 and Annex 2);
- to review and prepare specifications for selected food additives and flavouring agents (sections 3 and 4 and Annex 2).

2.1 Modification of the agenda

Annatto extracts were scheduled for evaluation at a future meeting, when the results of toxicological studies that were being performed would become available to the Committee for consideration. Amyloglucosidase from *Aspergillus oryzae*, var. had been included in the call for data erroneously.

Sodium ethyl *p*-hydroxybenzoate, sodium propyl *p*-hydroxybenzoate, sodium methyl *p*-hydroxybenzoate, calcium sulfite, sodium formate, calcium formate, synthetic γ -tocopherol, synthetic δ -tocopherol, calcium tartrate, sorbitan trioleate, dipotassium diphosphate and dimagnesium diphosphate have been removed from the draft Codex General Standard for Food Additives and were referred to the Committee for evaluation. There was no indication, however, that any of these substances are used as food additives, and consequently little information was provided that would permit the establishment of Acceptable Daily Intakes (ADIs) or the preparation of specifications. Phenyl salicylate was removed from the agenda because no data were available.

2.2 Principles governing the toxicological evaluation of compounds on the agenda

In making recommendations on the safety of food additives and contaminants, the Committee took into consideration the principles established and contained in Environmental Health Criteria, No. 70, *Principles for the safety assessment of food additives and contaminants in food* (Annex 1, reference 76), as well as the principles elaborated subsequently at a number of its meetings (Annex 1, references 77, 83, 88, 94, 101, 107, 116, 122, 131, 137, 143 and 149), including the present one. Environmental Health Criteria, No. 70 (Annex 1, reference 76) contains the most important observations, comments and recommendations made, up to the time of its publication, by the Committee and associated bodies in their reports on the safety assessment of food additives and contaminants. At its present meeting, the Committee noted that the publication included recommendations that are still appropriate and indicated potential problems associated with those that are no longer valid in the light of technological changes.

2.3 Principles for the safety assessment of chemicals in food

The Committee was informed that FAO and WHO are intending to update and consolidate principles and methods for the safety assessment of chemicals in food, including food additives, contaminants, and residues of veterinary drugs and pesticides. The project was

initiated on the basis of a recommendation of the Conference on International Food Trade Beyond 2000 that was held in Melbourne, Australia, in October 1999 (2), and in view of the scientific advances, changes in procedures and the increasing complexity of assessments of chemicals in food that have occurred since the publication of Environmental Health Criteria, No. 70 (Annex 1, reference 76) and Environmental Health Criteria, No. 104, *Principles for the toxicological assessment of pesticide residues in food* (3). The project would include consideration of all those aspects of the assessment of chemicals in food that are addressed by the Committee and by the Joint FAO/WHO Meeting on Pesticide Residues.

The Committee recognized the importance of this initiative and recommended that it be undertaken as soon as possible.

2.4 Flavouring agents evaluated by the Procedure for the Safety Evaluation of Flavouring Agents

The Committee questioned whether some of the substances included in the lists of flavouring agents that it had been asked to evaluate at its present meeting were in fact used as flavouring agents. The Committee noted that some of the substances were used extensively in food processing as solvents, emulsifiers or preservatives.

The Committee stressed that the Procedure for the Safety Evaluation of Flavouring Agents is intended for application to flavouring agents used to impart flavour to foods and not to other uses of these substances or to other chemicals that may be used in flavouring formulations. Consequently, the Committee was unable to finalize the evaluations of certain substances listed on the agenda,¹ pending confirmation of their use and intake as flavouring agents.

A clear definition of “flavouring agent” has not been elaborated by the Committee. Although Environmental Health Criteria, No. 70 (Annex 1, reference 76) provides some guidance, the Committee recommended that this issue be addressed at a future meeting.

2.5 α,β -Unsaturated carbonyl compounds and aldehydes

The α,β -unsaturated carbonyl group is a reactive moiety that represents a potential structural alert for toxicity. Five flavouring agents containing such a group were considered by the Committee at its forty-ninth meeting (Annex 1, reference 131), but their evaluation was postponed, pending consideration of other α,β -unsaturated carbonyl compounds. The safety of these five agents was reconsidered by

¹ See sections 4.1.3–4.1.5.

the Committee at its fifty-fifth meeting (Annex 1, reference 149), when it also evaluated furfural, cinnamaldehyde, structural analogues of cinnamaldehyde, pulegone and esters of the corresponding alcohols, which are predicted to be metabolized by formation of α,β -unsaturated carbonyls. The available data on the toxicity of these compounds in experimental animals showed a number of adverse effects at high doses, and no-observed-effect-levels (NOELs) for these effects were identified. The presence of protective processes in cells, such as conjugation with glutathione, provides adequate capacity for detoxification at the low doses associated with the use of such compounds as flavouring agents. In consequence, the Committee concluded that the presence of an α,β -unsaturated carbonyl group in a flavouring agent, or its formation during metabolism, would not preclude assessment of that substance by the Procedure for the Safety Evaluation of Flavouring Agents. This conclusion was supported by data on the toxicokinetics of 4-phenyl-3-buten-2-one (No. 820), which was considered by the Committee at its present meeting. This α,β -unsaturated carbonyl compound undergoes complete first-pass metabolism in rats and mice after oral administration and is rapidly eliminated (with a half-life of 20 min in rats and 10 min in mice) after intravenous administration. A number of other α,β -unsaturated carbonyl compounds were also evaluated by the Committee at its present meeting (Nos 821, 826 and 829), as were several compounds predicted to be metabolized to an α,β -unsaturated carbonyl compound (Nos 819, 944, 946 and 948).

Aldehyde groups are also chemically reactive and can bind to soluble proteins and protein components of membranes. Several aldehydes were evaluated previously by the Committee, and the potential genotoxicity of furfural was considered in detail at the fifty-first meeting (Annex 1, reference 137). Furfural was reported to be genotoxic in three of 16 assays for reverse mutation in *Salmonella typhimurium* and in one of three assays for *rec* gene mutation in *Bacillus subtilis*. A few chromosomal aberrations were seen in Chinese hamster ovary cells in culture when furfural was added at relatively high concentrations (Annex 1, reference 138). Sister chromatid exchanges and forward mutations were induced in mouse lymphoma cells. The Committee concluded that the weak activity seen in vitro in some tests for genotoxicity might be explained by the reactivity of the aldehyde group. Various metabolic processes (i.e. oxidation, conjugation and condensation) effectively eliminate the reactive aldehyde functional group, when the metabolic pathways are not saturated by high, non-physiological doses. The flavouring agents evaluated at the present meeting included a number of aldehydes (Nos 22, 865, 866,

868, 869, 877–879, 889–893, 896–898 and 937) and compounds that are predicted to be metabolized to aldehydes, such as acetals (Nos 837–840, 867, 940–949 and 954). Metabolism of these flavouring agents is predicted to result in gradual formation of aldehydes, which undergo extensive biotransformation, resulting in only low concentrations of the aldehydes per se. The results of tests for reverse mutation in bacteria were positive for pyruvaldehyde (No. 937), but consistently negative for Nos 22, 80, 95, 98, 867, 868, 877–879, 889, 893, 896, 897 and 953; the results of assays for *rec* gene mutation in *B. subtilis* were negative (Nos 878, 889 and 893) or equivocal (Nos 22 and 896). Chromosomal aberrations were reported in vitro in some studies with Nos 22, 878, 889, 893, 896 and 937, but not with No. 80. Similarly, sister chromatid exchanges were reported in some studies with Nos 22, 80, 878, 889 and 937, but not with Nos 868, 893 and 897. Mutations were reported in mouse lymphoma cells exposed to some aldehydes (Nos 80, 877, 878 and 893), but not other aldehydes or acetate (Nos 867, 889 and 896). The results of studies in vivo did not indicate genotoxicity after oral administration in a variety of test systems: in *Drosophila melanogaster* (with Nos 22, 80, 879 and 893), in assays for micronucleus formation in mice (with Nos 879, 889 and 893) and in assays for dominant lethal mutations in mice (No. 896). Sister chromatid exchange was induced in mice and hamsters by intraperitoneal injection of acetaldehyde (No. 80), and weakly positive results were obtained in several tests in vivo with pyruvaldehyde (No. 937) at very high doses (>200 mg/kg of body weight). Pyruvaldehyde is a natural component of some foods, and the amount ingested due to its use as a flavouring agent would be much less than the estimated intake from natural sources. The Committee concluded that metabolic processes such as oxidation and conjugation effectively eliminate reactive aldehyde functional groups from such substances when they are consumed in the amounts that would arise from their use as flavouring agents.

2.6 Minimum assay values for flavouring agents

At its fifty-third meeting, the Committee developed criteria for establishing specifications for flavouring agents (Annex 1, reference 143). The Committee noted that these criteria — chemical formula and relative molecular mass, identity test and the minimum amount that can be determined (minimum assay value) — constitute the core information required to establish acceptable specifications. At its present meeting, the Committee considered that a minimum assay value of 95% for an individual flavouring agent would apply to both the flavouring agent itself and to the agent plus its known secondary components. The minimum assay values of about 90% of the flavouring agents evaluated to date meet or exceed 95%, and the Committee received

information about the nature of the secondary components of the others. The Committee noted that 95% is not a fixed value for the acceptability of specifications for flavouring agents and that flexibility can be used in establishing an acceptable level of secondary components, taking into account the probable levels of intake and other considerations.

Many of the secondary components are structurally related to the named flavouring agents. They typically comprise small amounts of starting materials, isomers and other flavouring agents. As these secondary components share many of the properties of the named flavouring agents, and in some cases are metabolites, their safety would not be expected to present a concern or can be evaluated from appropriate data on metabolism and toxicity.

The Committee noted that, in applying the Procedure for the Safety Evaluation of Flavouring Agents, information on secondary components included in the specifications should be considered with data on intake and on the potential toxicity of the flavouring agent and its structural analogues. The Committee therefore recommended that data on specifications be submitted before or at the same time as all other information necessary for evaluating the safety of a flavouring agent.

2.7 Requests for data relating to intake assessments

The Committee recognized that it is unnecessary to request data for assessing intake for all the substances on its agenda, as it had done recently, and developed criteria for determining when such information would be needed. These are described below. In general, calls for data should specify the information required for each substance on the agenda, as the data required for evaluating food additives are different from those required for evaluating contaminants.

2.7.1 Food additives

Data for assessing intake should be requested in the case of food additives that are being evaluated for the first time or are being re-evaluated, except for food additives:

- for which only specifications are to be considered;
- for which the Committee has recently deferred an evaluation, pending the provision of a specific toxicological study or information on specifications, provided it has evaluated intake of the additive during the preceding 3–5 years.

Information on proposed maximum levels should be provided in the call for data for food additives included in the draft Codex General

Standard for Food Additives, so that national assessments of intake based on such maximum levels, national maximum levels and/or actual levels of use can be submitted. The Committee has formulated data sheets for submission of national data, which are included in the guidelines for the preparation of working papers on the intake of food additives.

2.7.2 Contaminants

For contaminants, an intake assessment is required in all cases. The call for data should request data on:

- the occurrence and concentrations of the contaminant (both individual and summary data) from all available sources, preferably submitted on data sheets, with information on sampling and analytical techniques, data quality and reliability, reporting conventions and appropriate processing factors;
- national intake of the contaminant derived from national surveys of food consumption and concentrations.

2.8 Principles governing the establishment and revision of specifications

2.8.1 Inclusion of raw materials and manufacturing methods in specifications

Principles for the safety assessment of food additives and contaminants in food (Annex 1, reference 76) states that “to establish the chemical identities of additives, it is necessary to know the nature of the raw materials, methods of manufacture and impurities. This information is used to assess the completeness of analytical data on the composition of additives, and to assess the similarity of materials used in biological testing with those commercially produced.”

As there are increasing volumes of food additives in international trade, specifications must include brief descriptions of the raw materials and methods of manufacture used, excluding proprietary details, in order to provide a full account of the product being evaluated. If this information is not available, the Committee cannot know whether the products being evaluated were produced from materials or methods that are different from those in the specifications; consequently, impurities might have arisen that were not considered during the toxicological evaluation. The level of detail of the descriptions should be similar to that in specifications elaborated by the Committee for additives made by fermentation or from plant materials.

2.8.2 **General specifications and considerations for enzyme preparations used in food processing**

The Committee has, on many occasions, addressed issues related to specifications for enzyme preparations used in food processing. The general specifications currently in use for enzymes were first elaborated by the Committee at its twenty-sixth meeting (Annex 1, reference 59). Several revisions have been made, including the following:

- an addendum to address issues arising from use of enzymes from genetically modified microorganisms (Annex 1, references 94, 96, 137 and 139);
- addition of an appendix to describe the method for determining antimicrobial activity (Annex 1, reference 58);
- an amendment to address inclusion of microbial strain numbers in the specifications for enzyme preparations (Annex 1, reference 139);
- addition of the general requirement that source microorganisms be non-pathogenic and non-toxicogenic (Annex 1, reference 145).

At its fifty-fifth meeting (Annex 1, reference 149), the Committee reiterated its view, expressed at its fifty-third meeting (Annex 1, reference 145), that Annex 1 (General specifications for enzyme preparations used in food processing) of the *Compendium of food additive specifications* (Annex 1, reference 96) required updating in the light of technological developments and to ensure consistency and coherence with the appendices, including Appendix B (General considerations and specifications for enzymes from genetically manipulated microorganisms).

At its present meeting, the Committee noted that the revised general specifications require that all new enzyme preparations undergo a general safety assessment. Many of the requirements previously outlined for enzyme preparations from genetically modified microorganisms are appropriate for all preparations, regardless of source, and the Committee revised the general specifications to reflect those requirements. For enzymes from genetically modified sources, information is now required on the microbial strain used as the source organism and the genetic material introduced into and remaining in the final microbial strain used in production.

At its present meeting, the Committee noted that the list of mycotoxins contained in the existing general specifications was not relevant to all food enzyme preparations from fungal sources. It agreed that an attempt to list all known mycotoxins of potential concern was impractical and unwarranted. The Committee further agreed that enzyme preparations derived from fungal sources be evaluated for those mycotoxins that are known to be synthesized by strains of the species or related species used in the production of the enzyme preparation.

With regard to limits on heavy metals, the Committee agreed that the specifications for lead contained in the existing general specifications should be lowered from 10mg/kg to 5mg/kg. The Committee recognized that arsenic is not a concern in enzyme preparations, and therefore deleted the limit for this metal. Moreover, as there is no traceable source of cadmium or mercury in enzyme preparations, the Committee saw no need to establish limits for those metals. Such changes are consistent with the Committee's current policy on heavy metals (Annex 1, reference 145).

In considering microbiological contamination of enzyme preparations, the Committee agreed that the existing microbiological criteria (for *Salmonella* spp., *Escherichia coli* and total coliforms) and the requirement that use of preparations should not increase the total microbial count in treated food above the threshold considered to be acceptable for the respective foods are sufficient to ensure microbial safety. The criteria and the requirement were thus retained. The Committee noted that the specification for a total viable count of 5×10^4 organisms per gram included in the existing general specifications did not provide an indication of the safety of an enzyme preparation. It was therefore deleted.

In considering allergenic potential, the Committee emphasized that, when the source organism of an enzyme preparation is a genetically modified microorganism, the necessity for evaluating the allergenic potential of the gene products encoded by the inserted DNA should be assessed. The Committee agreed that, when the DNA sequence of an enzyme from a genetically modified production microorganism is comparable to that coding for an enzyme already known to have a history of safe use in food, there would be no need to assess the allergenic potential of that enzyme further.

Finally, the Committee recognized that the revised specifications include many criteria for safety evaluation that would be more appropriately listed elsewhere. The Committee strongly recommended that *Principles for the safety assessment of food additives and contaminants in food* (Annex 1, reference 76) be revised to include the safety assessment of enzymes intended for use in food and that such guidelines should subsequently be removed from the general specifications.

3. **Specific food additives (other than flavouring agents)**

The Committee evaluated two food additives for the first time and re-evaluated a number of others. Information on the safety evaluations

and on specifications is summarized in Annex 2. Details of further toxicological studies and other information required for certain substances are given in Annex 3.

3.1 **Safety evaluations**

3.1.1 **Emulsifiers**

3.1.1.1 *Diacetyltartaric and fatty acid esters of glycerol*

Diacetyltartaric and fatty acid esters of glycerol were reviewed by the Committee at its tenth and seventeenth meetings (Annex 1, references 13 and 32). At its seventeenth meeting, the Committee allocated an ADI of 0–50 mg/kg of body weight on the basis of the results of biochemical and metabolic studies and feeding tests in animals. At the same meeting, the Committee also reviewed mixed tartaric, acetic and fatty acid esters of glycerol and allocated an ADI “not limited”, with the provision that the total intake of tartaric acid from food additives should not exceed 30 mg/kg of body weight per day.

Specifications established by the Committee at its fifty-first meeting (Annex 1, reference 137) covered both the above-mentioned products under the name “diacetyltartaric and fatty acid esters of glycerol”, as the Committee was aware that the two products could not be distinguished analytically. At that meeting, the Committee recommended that the material defined in the specifications be evaluated toxicologically. At its present meeting, the Committee considered the data that were available previously as well as newly submitted information.

The diacetyltartaric and fatty acid esters of glycerol consist of mixed glycerol esters of mono- and diacetyltartaric acid and fatty acids of food fats. They can be manufactured either by the interaction of diacetyltartaric anhydride and mono- and diglycerides of fatty acids in the presence of acetic acid, or by the interaction of acetic anhydride and mono- and diglycerides of fatty acids in the presence of tartaric acid.

Owing to inter- and intramolecular exchange of acyl groups, the two methods of production result in essentially the same components, the distribution of which depends on the relative proportions of the basic raw materials, on temperature and on reaction time. Diacetyltartaric and fatty acid esters of glycerol may contain small amounts of free glycerol, free fatty acids and free tartaric and acetic acids. They may be further specified as to the acid value, total tartaric acid content, free acetic acid content, saponification value, iodine value, free fatty acid content and the solidification point of the free fatty acids.

The draft Codex General Standard for Food Additives includes use of diacetyltartaric and fatty acid esters of glycerol as an emulsifier, sequestrant and stabilizing agent in a wide range of foods at a maximum concentration of 10 g/kg.

Biological data. Biochemical studies suggest that diacetyltartaric and fatty acid esters of glycerol are hydrolysed in the gastrointestinal tract to yield mono- and diglycerides and acetylated tartaric acid. As mono- and diglycerides are natural dietary constituents, they would be subjected to natural digestion and absorption processes. Diacetyltartaric acid is not a natural constituent of the diet, and there is evidence that it may be further hydrolysed to yield acetic and tartaric acids. When labelled diacetyltartaric and fatty acid esters of glycerol were administered to rats, only about one-third of a ^{14}C label on tartaric acid was absorbed; slightly more was excreted in expired air than in urine.

The studies reviewed previously indicated little toxicity after administration of a single oral dose of diacetyltartaric and fatty acid esters of glycerol. Three studies of the potential long-term toxicity of this product when given in the diet to small numbers of rats showed no adverse effects of dietary concentrations of up to 200 g/kg on mortality rate, physical appearance, body weight, food consumption, reproduction or the histological appearance of the main organs. Dogs also showed no adverse effects when fed diets containing concentrations of up to 200 g/kg for more than 2 years.

The information reviewed for the first time at the present meeting consisted of a 2-week study of palatability, a long-term study of toxicity and carcinogenicity, a two-generation study of reproductive toxicity and a study of developmental toxicity, all conducted in rats, plus two studies of genotoxicity, for point mutations in bacteria and for clastogenicity in isolated human lymphocytes. In addition, a 6-month study was conducted in male rats to elucidate some of the effects seen in the long-term study of toxicity and carcinogenicity.

In the short- and long-term studies in rats, diacetyltartaric and fatty acid esters of glycerol at a concentration of 100 g/kg of diet caused a transient occurrence of soft stools, particularly in males. Food consumption was frequently depressed at this concentration, most consistently during the first weeks of treatment. The palatability of the diet was not improved by incorporating diacetyltartaric and fatty acid esters of glycerol into breadcrumbs or by volatilizing the fatty acids in the substance by freeze-drying the diet before administration. Body-weight gain tended to be reduced, but this effect was not observed consistently in the short-term study. In the long-term study, consumption of a diet containing diacetyltartaric and fatty acid esters of glycerol at 100 g/kg was associated with decreased body weight. This effect was transient in male rats, but body weights more than 10% lower than those of controls persisted in females into the second year of the study. Supplementing the diet with additional protein,

magnesium, pyridoxine (vitamin B₆) and cyanocobalamin (vitamin B₁₂) reduced the decrease in weight observed with the compound at 100 g/kg of diet in the 6-month study.

Administration of diacetyltartaric and fatty acid esters of glycerol was associated with a decrease in the proportion of lymphocytes and an increase in the proportion of neutrophils in the total leukocyte count during the 6-month study and during the first year of the long-term study. However, these effects were transient and dependent on the type of diet.

In the 6-month study, inclusion of diacetyltartaric and fatty acid esters of glycerol at a concentration of 60 or 100 g/kg of diet was associated with an increase in the urinary excretion of calcium. In the long-term study, differences in the weights of the adrenal glands, kidneys and spleen were observed after 1 year of treatment but were no longer evident after 2 years. Males fed a diet containing 100 g/kg showed an increase in both the incidence and the severity of mineralization in the kidney papilla and pelvis after 1 and 2 years of treatment. Administration at 100 g/kg of diet for 2 years resulted in an increased prevalence of microscopic abscesses in the kidneys of males and an increased severity of nephrocalcinosis in females.

After 2 years of treatment, a dose-related increase in the incidence of adrenal medullary adenomas was seen in males, affecting 4/50, 6/50, 11/50 and 15/50 (statistically significant) animals at 0, 30, 60 and 100 g/kg of diet, respectively, and 1/50 and 4/50 females at 0 and 100 g/kg of diet, respectively. Focal medullary hyperplasia was observed in 3/50, 10/48 (statistically significant), 15/50 (statistically significant) and 15/50 (statistically significant) males at 0, 30, 60 and 100 g/kg of diet, respectively, and in 0/50 and 9/50 (statistically significant) females at 0 and 100 g/kg of diet, respectively.

Statistically significantly higher incidences of haemangioma and haemorrhage in the mesenteric lymph nodes were observed in males fed diets containing 100 g/kg of diacetyltartaric and fatty acid esters of glycerol, while the incidence of sinus histiocytosis of the mesenteric lymph nodes was statistically significantly increased in all treated males.

Myocardial fibrosis was observed more frequently in males at the highest dietary concentration (13/50) than in the control group (3/50). Females at the highest concentration had higher incidences of endometrial hyperplasia (7/50 vs 0/50) and cystic endometrial hyperplasia (14/50 vs 7/50) than controls at the end of the study. Histopathological examinations were carried out on the hearts of only

some of the males and on the uteri of only some of the females at the two lower doses.

In a two-generation study of reproductive toxicity, parental males of the F₀ generation ate less of the diet containing 100 g/kg of diacetyltartaric and fatty acid esters of glycerol and gained less weight during the pre-mating period. Although F₀ females at this dietary concentration also ate less food during the first few weeks of the study, their body-weight gains were not affected. The body weights, body-weight gains and food consumption of the F₁ adults were unchanged. The survival of the F₁ and F₂ litters was not affected by treatment. The weight gains during lactation of the F₁ generation litters of dams given diacetyltartaric and fatty acid esters of glycerol at concentrations of 60 or 100 g/kg of diet and of the F₂ generation litters of dams at 100 g/kg of diet were significantly reduced. The reproductive organs were not assessed histologically. The NOEL for reproductive toxicity was 30 g/kg of diet, equivalent to 1500 g/kg of body weight per day.

Evaluation. High dietary concentrations of diacetyltartaric and fatty acid esters of glycerol were associated with decreased body weights in adult rats and their offspring, but it could not be ascertained from the available data whether these decreases were secondary to or independent of decreased food consumption.

In the 2-year study in rats, the groups treated with diacetyltartaric and fatty acid esters of glycerol were apparently compared with controls fed diets containing monoglyceride. In order to assess whether some of the adverse effects were treatment-related, it would be necessary to compare the effects in treated groups with those in both untreated and monoglyceride-treated control groups, and to compare the control groups with one another. In the absence of additional data on the incidence of myocardial fibrosis and adrenal medullary hyperplasia in animals at the lowest and intermediate doses, no NOEL could be identified in the long-term study. The previous ADI of 0–50 mg/kg of body weight was made temporary until 2003, pending submission of all the necessary additional information.

The specifications for diacetyltartaric and fatty acid esters of glycerol were revised. As specifications no longer exist for tartaric, acetic and fatty acid esters of glycerol, mixed, the previous ADI is no longer applicable and was withdrawn.

A toxicological monograph was prepared, incorporating information from the earlier monographs and summaries of the studies reviewed for the first time at the present meeting.

3.1.1.2 *Quillaia extracts*

Quillaia extracts (synonyms: bois de Panama, Panama bark extracts, quillai extracts, Quillay bark extracts, soapbark extracts) are obtained by aqueous extraction of the milled inner bark or wood of pruned stems and branches of *Quillaja saponaria* Molina (family Rosaceae), which is a large evergreen with shiny, leathery leaves and a thick bark, native to China and several South American countries, principally Bolivia, Chile and Peru. The term “quillaia” refers to the dried inner bark of the tree.

Unpurified extracts contain over 60 triterpenoid saponins, consisting predominantly of glycosides of quillaic acid. Polyphenols and tannins are major components. Some simple sugars and calcium oxalate are also present. The saponin concentration of freshly prepared, unpurified extracts is 190–200 g/kg of solids (about 20%). The extracts are treated with “stabilizing agents” such as egg albumin and polyvinylpyrrolidone and then filtered through diatomaceous earth. The stabilizing agents remove substances that would probably precipitate during storage, such as protein–polyphenol complexes. After filtration, the liquid is concentrated, and the concentrate may be sold as such (solids constituting about 550 g/l) or be spray-dried and sold as a powder containing carriers such as lactose and maltodextrin. The unpurified extracts are used in food applications, primarily for their foaming properties.

Semi-purified powdered extracts are produced by subjecting unpurified extracts to ultra-filtration or affinity chromatography to remove most solids other than saponins, such as polyphenols. These semi-purified extracts have higher saponin concentrations (750–800 g/kg of solids; about 80%) and better emulsifying properties than unpurified extracts.

Highly purified extracts are produced for use as adjuvants in the production of animal and human vaccines and not for food use. These products generally contain more than 90% saponins.

In previous evaluations, the Committee considered data on unpurified quillaia extracts. Quillaia extracts were reviewed toxicologically by the Committee at its twenty-sixth meeting (Annex 1, reference 59). The available toxicological data included adequate lifetime studies in mice and rats, from which a NOEL was identified. However, in the absence of data, no specifications were prepared, and, hence, no ADI could be allocated. At its twenty-ninth meeting (Annex 1, reference 70), the Committee prepared new tentative specifications and established an ADI of 0–5 mg/kg of body weight. The present evaluation was conducted in response to a request by the Codex Committee on Food Additives and Contaminants at its Thirty-

second Session (4) that the Expert Committee re-evaluate all relevant information on the toxicity and, in particular, the intake of quillaia extracts. No new data were submitted to the Committee at its present meeting. The Committee evaluated published reports on quillaia extracts or specific saponins that provided information relevant to a toxicological assessment of quillaia extracts.

Biological data. Quillaia extracts are mixtures of biologically active compounds that include saponins, tannins, polyphenols and calcium oxalate. The saponins present in quillaia extracts have a variety of biological activities: they are haemolytic, cytotoxic, enhance immune reactions, cause mucosal irritation and inflammation and are anti-hypercholesterolaemic. The biological activities and the potency of individual saponins vary widely and depend primarily on the route of administration.

Studies of acute toxicity showed that quillaia extracts are less toxic when administered orally than when administered subcutaneously or intravenously. Fractions isolated from *Q. saponaria* differed widely in acute toxicity as well as in adjuvant activity and cholesterol-binding capacity. QS-18, the major saponin of quillaia extracts, was more acutely toxic to mice than two other saponins that were isolated and was more toxic than the extract itself when administered intradermally.

In a 90-day study, rats were fed diets containing 40g/kg quillaia extract (equivalent to 2000mg/kg of body weight per day). The specifications of the preparation conformed to the Emulsifiers and Stabilisers in Food Regulations 1975 of the United Kingdom, but information on the actual composition of the material tested was not available. The animals showed decreased body-weight gain, decreased weight of the liver relative to body weight and increased stomach weight, with no treatment-related histological changes. The NOEL was 6 g/kg of diet, equivalent to 400mg/kg of body weight per day.

In a more recent 90-day study, rats were given quillaia saponins in deionized water by gavage at a dose of 1200mg/kg of body weight. Severe and lethal toxic effects were observed during the study. In the surviving animals, the weights of several organs were increased, and several haematological and clinical parameters were changed. Histo-pathological examination showed inflammatory changes in the fore-stomach, larynx, trachea and lungs.

Minor changes in body-weight gain and in the relative weights of some organs were reported in lifetime studies in mice and rats given

quillaia extracts (with specifications conforming to the Emulsifiers and Stabilisers in Food Regulations 1975 of the United Kingdom), at dietary concentrations of up to 30 g/kg for mice and 15 g/kg for rats. No compound-related histopathological changes were reported. The NOELs for quillaia extracts in the diet were 5 g/kg (equivalent to 700 mg/kg of body weight per day) for mice and 10 g/kg (equivalent to 500 mg/kg of body weight per day) for rats.

The Committee noted that the differences in toxicity observed in the 90-day studies in rats treated orally, outlined above, might have been due to differences in the concentrations and types of saponins present in the test material and/or differences in the method of administration, i.e. in the diet and by gavage in water.

Evaluation. The existing specifications for quillaia extracts were revised in order to clarify the differences between unpurified and semi-purified extracts. As additional information on composition was determined to be necessary, the specifications were designated as tentative. Once the requested information has been received, the Committee will consider whether separate specifications for unpurified and semi-purified extracts are required.

The Committee decided that the previously established ADI of 0–5 mg/kg of body weight for unpurified extracts should be made temporary and extended it until 2003, pending clarification of the specifications for quillaia extracts; further studies of toxicity with specified quillaia products similar to the product consumed by humans may be required. The Committee emphasized that the temporary ADI is not applicable to the semi-purified extract or to any other product derived from *Q. saponaria* or from other species of *Quillaia*.

Assessment of intake. Quillaia extracts can be used as foaming agents in soft drinks and cocktail mixes and as emulsifiers in foods such as baked goods, sweets, frozen dairy products, gelatine and puddings. Their major food use is in soft drinks such as ginger beer, root beer and cream soda.

Calculations based on the temporary ADI of 0–5 mg/kg of body weight and the assumption that quillaia extracts are used in soft drinks at a concentration of 500 mg/kg indicated that a person weighing 60 kg could drink up to 600 g/day of a soft drink before exceeding the ADI, while a child weighing 15 kg could drink only 150 g/day of a soft drink before exceeding the ADI. Data on food consumption submitted to the Committee indicated that consumers in Australia who are at the 95th percentile of the distribution of consumption of soft drinks that are likely to contain the additive and children aged 1.5–4 years in the United Kingdom who consume soft drinks at the

97.5th percentile could exceed these amounts. However, these calculations may have overestimated long-term consumption because the data were derived from short-term surveys.

Estimates of intake based on consumption of soft drinks likely to contain this food additive and the levels of use of quillaia extracts permitted in the draft Codex General Standard for Food Additives were submitted by Australia and the USA. Estimates of the mean intake in the United Kingdom were also available, which were based on consumption of all water-based flavoured drinks and are therefore conservative. In Australia, the mean intakes were 3 mg/kg of body weight per day (60% of the ADI) for consumers of drinks containing the additive at the level permitted in the draft Codex General Standard for Food Additives (500 mg/kg) and 7.2 mg/kg of body weight per day (145% of the ADI) for persons at the 95th percentile of consumption. In the USA, the estimated mean intakes of quillaia extracts were 1.5 mg/kg of body weight per day (30% of the ADI) for consumers of drinks containing the additive at the level permitted in the draft Codex General Standard for Food Additives and 2.7 mg/kg of body weight per day (54% of the ADI) for consumers at the 90th percentile.

Estimates of intake based only on consumption of soft drinks likely to contain the food additive and national levels of use were submitted by the USA. The maximum level of use of quillaia extracts by manufacturers in the USA is 100 mg/kg. The estimated mean intake by consumers was 0.3 mg/kg of body weight per day (6% of the ADI), and that by consumers at the 90th percentile was 0.54 mg/kg of body weight per day (11% of the ADI). Data from the United Kingdom, based on a level of use by manufacturers of 95 mg/kg, indicated that children who consume soft drinks at the 97.5th percentile level would consume quillaia extracts at 5.2 mg/kg of body weight per day (105% of the ADI), but this value may be an overestimate of intake as it is based on consumption of all water-based flavoured drinks.

Use at a maximum level of 95–100 mg/day (that reported by the manufacturers), as in the United Kingdom and the USA, appeared to be adequate for the technological functioning of quillaia extracts as foaming agents in soft drinks and did not appear to result in intakes that exceed the ADI. Young children are a possible exception, but, as the results of a short-term nutritional survey were used, the frequency or duration of their potential excursion above the ADI could not be determined.

The Committee recommended that the Codex Committee on Food Additives and Contaminants review the use of quillaia extracts at

500mg/kg proposed in the draft Codex General Standard for Food Additives.

A toxicological monograph was prepared.

3.1.2 **Enzyme preparation**

3.1.2.1 *Invertase from *Saccharomyces cerevisiae**

Invertase from *Saccharomyces cerevisiae*, or “bakers’ yeast”, hydrolyses sucrose to a mixture of glucose and fructose (invert sugar). This substance was reviewed by the Committee at its fifteenth meeting (Annex 1, reference 26) as one of the active principles of carbohydrase from *Saccharomyces* species. It is produced by controlled, submerged fermentation of a pure culture of *S. cerevisiae*. At the end of fermentation, the yeast cells are collected, washed and subjected to autolysis. The lysate is centrifuged and/or filtered to remove cell debris. The resulting enzyme preparation may be dried or ultra-filtered to a desired enzyme concentration. Liquid ultra-filtered products can be treated further with activated charcoal to remove colour and then filtered under sterile conditions. The invertase product is standardized with food-grade diluents.

At its fifteenth meeting (Annex 1, reference 26), the Committee concluded that enzymes derived from microorganisms that are traditionally accepted as constituents of foods or are normally used in the preparation of foods should themselves be regarded as foods. Invertase from *S. cerevisiae* was evaluated at the present meeting because it was being considered for inclusion in the draft Codex General Standard for Food Additives.

Invertase is fundamental to the manufacture of soft-filled chocolates and liquid-centre confectionery, there being no additive that fulfils the same technological function. In the filling for chocolates, invertase is used at a concentration of 1 g/kg of sucrose, resulting in a concentration of 0.6 g/kg (600 mg/kg) in the finished product.

The intake of invertase predicted by the Scientific Committee on Food of the Commission of the European Union was 15 mg/day, assuming consumption of 25 g of filled chocolates per day out of a total of 50 g of chocolate of all types and a concentration of invertase of 600 mg/kg of chocolate.

The potential intake of invertase from its use in chocolates was also predicted for the Australian population from individual dietary records obtained in a survey in 1995. Assuming consumption of 600 mg/kg of filled chocolate, the mean invertase intake by consumers was 20 mg/day (33 g/day of filled chocolate), and that of persons at the 95th percentile of consumption was 61 mg/day (100 g/day of filled choco-

late). These estimates were based on 24-h recalls of food consumption, which tend to result in overestimates of consumption on a long-term basis. The intake of invertase by young children and adolescents was similar to that of adults but would be relatively higher than that of the general population if expressed per kilogram of body weight.

No biological data were available. *S. cerevisiae* has a well-established history of use in fermented foods, including bread, alcoholic beverages, some milk products and cocoa. In line with the general principles outlined in *Principles for the safety assessment of food additives and contaminants in food* (Annex 1, reference 76), invertase from *S. cerevisiae* that meets the specifications developed at the present meeting was considered to be acceptable, as *S. cerevisiae* is commonly used in the preparation of food. Its use should be limited by good manufacturing practice.

A toxicological monograph was not prepared. New specifications were prepared.

3.1.3 **Food colours**

3.1.3.1 *β*-Carotene from *Blakeslea trispora*

The Committee did not undertake a general re-evaluation of β -carotene for use as a colouring agent but focused its assessment on the production and analytical characteristics of β -carotene produced from *Blakeslea trispora*.

β -Carotene is obtained from *B. trispora* by co-fermentation of the two sexual types of the fungus in specific proportions. Both types are stable in cultures maintained under conditions consistent with good manufacturing practice. These source organisms are neither pathogenic nor toxinogenic. The compound is isolated from the fungal biomass by solvent extraction and crystallized. The main articles of commerce are suspensions in food-grade vegetable or plant oil and water-dispersible powders, which are easy to use and improve stability, as carotenes readily undergo oxidation.

As in synthetic β -carotene, the colouring principle of β -carotene from *B. trispora* consists predominantly of the all-*trans* isomer of β -carotene. The content of total colouring matter is not less than 96% (expressed as β -carotene). β -Carotene from *B. trispora* may also contain other carotenoids, of which γ -carotene accounts for the major part, at concentrations up to 3%. These molecules occur naturally in carotenoid-containing vegetables.

The Committee considered that the source organisms, the production process and the composition of β -carotene from *B. trispora* do not

raise specific concerns and that the material should be considered toxicologically equivalent to chemically synthesized β -carotene, for which an ADI of 0–5 mg/kg of body weight was established by the Committee at its eighteenth meeting (Annex 1, reference 35). This opinion was given further credence by the negative results obtained in two tests for genotoxicity (mutagenicity and chromosomal aberration) considered at the present meeting. Therefore, the Committee established a group ADI of 0–5 mg/kg of body weight for synthetic β -carotene and β -carotene derived from *B. trispora*. The ADI relates strictly to use of β -carotene as a food colouring agent and not to its use as a food supplement.

Use of this preparation is unlikely to result in increased use of β -carotene as a food colour because the material is expected to be substituted for synthetic β -carotene.

A toxicological monograph was prepared. New specifications were prepared and designated as tentative, pending information on a suitable method for determination of residual ethyl acetate and isobutyl acetate used as solvents. This information is required by 2003.

3.1.3.2 Curcumin

Curcumin is obtained by solvent extraction of turmeric, which is in turn derived from ground rhizomes of *Curcuma longa* L. (*C. domestica* Valetton). In order to obtain concentrated curcumin powder, the extract is purified by crystallization. The commercial product consists predominantly of curcumins: the colouring principle (1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione) and its desmethoxy and bisdesmethoxy derivatives, in varying proportions. Minor amounts of oils and resins that occur naturally in turmeric may be present.

Turmeric oleoresin and curcumin, the main colouring component of turmeric oleoresin, were evaluated by the Committee at its thirteenth, eighteenth, twenty-second, twenty-fourth, twenty-sixth, thirtieth, thirty-fifth, thirty-ninth, forty-fourth and fifty-first meetings (Annex 1, references 19, 35, 47, 53, 59, 73, 88, 101, 116 and 137). At its eighteenth meeting, the Committee established a temporary ADI of 0–0.1 mg/kg of body weight for curcumin on the basis of the ADI for turmeric oleoresin (0–2.5 mg/kg of body weight) and an assumed average concentration of 3% curcumin in turmeric. The temporary ADI for curcumin was extended at the twenty-second, twenty-fourth, twenty-sixth, thirtieth, thirty-fifth and thirty-ninth meetings of the Committee. At its thirty-ninth meeting, the Committee requested the results of studies of carcinogenicity in mice and rats fed turmeric

oleoresin and the results of a study of reproductive and developmental toxicity with curcumin.

At its forty-fourth meeting, the Committee evaluated the results of the studies of carcinogenicity in rats and mice given turmeric oleoresin containing 79–85% curcumin and new data on the biochemistry and genotoxicity of the compound. The Committee concluded that data on the developmental toxicity of curcumin were no longer required, but reiterated its request for a study of reproductive toxicity. On the basis of a NOEL of 220mg/kg of body weight per day in the study of carcinogenicity in mice and a safety factor of 200, the Committee increased the temporary ADI to 0–1 mg/kg of body weight and extended it, pending submission of the results of a study of reproductive toxicity with curcumin.

At its fifty-first meeting, the Committee evaluated the results of studies of fertility in rats and mice treated with turmeric oleoresin and concluded that they did not provide assurance that the potential reproductive effects of curcumin had been adequately investigated. The Committee again extended the temporary ADI, pending submission of the results of a study of reproductive toxicity with a substance that complied with the specifications for curcumin, for review in 2001.

The results of the requested study were not available to the Committee at its present meeting. Nevertheless, the Committee was informed that a multigeneration study of reproductive toxicity with a substance that complied with the specifications for curcumin was under way and would be completed within the next few months. In view of this information, the temporary ADI of 0–1 mg/kg of body weight for curcumin was extended until 2003, pending submission of the results of this study.

A toxicological monograph was not prepared. The existing specifications were revised, with minor changes.

3.1.4 **Food salts**

3.1.4.1 *Phosphates, diphosphates and polyphosphates*

Phosphates, diphosphates and polyphosphates were evaluated by the Committee at its sixth, seventh, eighth, ninth, thirteenth, fourteenth, seventeenth and twenty-sixth meetings (Annex 1, references 6–8, 11, 19, 22, 32 and 59). A maximum tolerable daily intake (MTDI) of 70mg/kg of body weight was established at the twenty-sixth meeting on the basis of the lowest concentration of phosphorus (6600mg/day) that caused nephrocalcinosis in rats. It was considered inappropriate to establish an ADI, as phosphorus (as phosphates) is an essential nutrient and an unavoidable constituent of food. The MTDI is

expressed as phosphorus and applies to the sum of phosphates naturally present in food and the phosphates derived from use of these food additives.

This MTDI was considered to cover a number of phosphate salts, according to the principle established by the Committee at its ninth, twenty-third and twenty-ninth meetings (Annex 1, references 11, 50 and 70) that the ADI (or MTDI) established for ionizable salts should be based on previously accepted recommendations for the constituent cations and anions. However, in this case, while an MTDI has been established for the class of phosphate salts, certain specific salts were not included because specifications were lacking and because information was not available to indicate whether they were being used as food-grade materials.

At its present meeting, the Committee established specifications for certain specific phosphate salts, pending further information, as indicated below.

- Calcium dihydrogen diphosphate is manufactured by calcination of calcium orthophosphate at a temperature of about 270°C, with a molar ratio of calcium:phosphorus of about 1:2. It is used in fine bakery wares at concentrations of up to 20 g/kg.
- Monomagnesium orthophosphate is manufactured by partial neutralization of phosphoric acid with magnesium oxide and drying the resultant product. It is used in fine bakery wares at concentrations of up to 20 g/kg. The Committee noted that this substance is more concisely denoted as monomagnesium phosphate. It therefore deleted the prefix “ortho” for the substance in the specifications. The specifications were made tentative, pending further information on loss on drying, loss on ignition, the test method for loss on ignition and the assay method for the dihydrate.
- Sodium calcium polyphosphate is manufactured by the fusion of sodium phosphate and calcium carbonate at temperatures greater than 1000°C. Phosphoric acid is neutralized with sodium and calcium hydroxides in a molar ratio of 5:1. The resulting mixture undergoes calcination and is cooled, milled and sieved. It is used in processed cheese and processed cheese analogues at concentrations of up to 20 g/kg.
- Trisodium diphosphate is manufactured by hydration and drying of mixtures of sodium diphosphate. It is used in meat products at concentrations of up to 5 g/kg. The specifications were made tentative, pending further information on loss on drying, loss on ignition, the test method for loss on ignition and the assay method for the monohydrate.

The Committee included these salts in the group MTDI for phosphates, diphosphates and polyphosphates.

A toxicological monograph was not prepared.

3.1.5 **Glazing agent**

3.1.5.1 *Hydrogenated poly-1-decene*

Hydrogenated poly-1-decene is obtained by catalytic hydrogenation of mixtures of trimers, tetramers, pentamers and hexamers of 1-decenes, produced by oligomerization of 1-decene in the presence of a catalyst. The product is purified by filtration through activated clay. Hydrogenated poly-1-decene consists of a mixture of branched isomeric hydrocarbons, predominantly with more than 30 carbon atoms. Minor amounts of molecules with fewer carbons may be present.

Hydrogenated poly-1-decene was first evaluated by the Committee at its forty-ninth meeting (Annex 1, reference 131) for use as a glazing and releasing agent. A 28-day range-finding study and a 90-day study in rats that were available at that time were considered inadequate to support use of this product as a food additive. Data were requested to demonstrate that the oily coats observed in rats fed hydrogenated poly-1-decene in the 90-day study were not the result of systemic absorption of the material. The Committee also requested that the results of a study demonstrating lack of absorption in humans be provided. In the absence of such data, the results of long-term studies of toxicity and reproductive toxicity and information on the metabolism, distribution and excretion of hydrogenated poly-1-decene would be required.

At its fifty-third meeting (Annex 1, reference 143), the Committee reviewed a study of the distribution and excretion of [³H]hydrogenated poly-1-decene conducted in rats. This study established that the oiliness of the fur observed within 1–6 h of administration of a bolus dose was associated with radiolabelled material originating from the anal region, which was spread by grooming. However, while the study indicated that very little hydrogenated poly-1-decene was absorbed after oral administration, it did not allow clear definition of the fate or disposition of any absorbed material. The Committee was therefore unable to establish an ADI and requested an adequate study of the absorption and deposition of hydrogenated poly-1-decene in order to determine whether further studies were required.

At its present meeting, the Committee re-evaluated the results of the study of the distribution and excretion of hydrogenated poly-1-decene. Although no additional studies on distribution and excretion were submitted, the Committee's attention was drawn to arguments

that supported the validity of the previous study. In addition, the Committee evaluated a study of the effect of hydrogenated poly-1-decene on the absorption, distribution and excretion of linoleic acid and glycerol trioleate that had been submitted for consideration.

The Committee also revised the existing specifications for hydrogenated poly-1-decene in order to take into account the decrease from 3% to 1.5% in the concentration of molecules with fewer than 30 carbon atoms in products on the market for food additive use.

In its re-evaluation, the Committee accepted that equivalent information can be obtained with ^3H and ^{14}C , provided that the label is located in a metabolically stable position, as was the case for [^3H]hydrogenated poly-1-decene. It also accepted that, for technical reasons, use of ^{14}C -labelled hydrogenated poly-1-decene might be less appropriate, as the synthetic ^{14}C -labelled compound might be different from the substance used in the studies of toxicity. The results of the study indicated that <1% of the dose of [^3H]hydrogenated poly-1-decene was absorbed from the gut. The absorbed radiolabel was present largely as $^3\text{H}_2\text{O}$, probably arising from tritium exchange between the labelled substance and body water. The Committee concluded that absorption of hydrogenated poly-1-decene was negligible. This conclusion was corroborated by the results of the 90-day study in rats, which provided no evidence of its accumulation in tissues. Furthermore, the revised specifications for the substance, which require that it contains a maximum of 1.5% of compounds with fewer than 30 carbon atoms should ensure that absorption of components of low relative molecular mass is kept to a minimum.

The Committee concluded that the available studies were adequate to assess the toxicity and safety of hydrogenated poly-1-decene. An ADI of 0–6 mg/kg of body weight was established on the basis of the NOEL of 550 mg/kg of body weight per day in the 90-day study in rats for effects on the condition of the fur, liver weight and histological appearance, and a safety factor of 100.

An additional study in rats submitted for consideration by the Committee suggested that hydrogenated poly-1-decene may decrease the bioavailability of linoleic acid, an essential fatty acid. However, the Committee concluded that a nutritionally relevant decrease in bioavailability would not occur if hydrogenated poly-1-decene was consumed at the level of the ADI, i.e. a maximum of 360 mg/person per day.

Hydrogenated poly-1-decene can be used as a release agent in bread prepared in commercial baking operations at concentrations of up to 300–500 mg/kg and in glazed fruit at concentrations of up to 2000 mg/kg. Bread is expected to be the major source of total intake of

this compound. If use only in bread is assumed, it can be calculated that up to 720g of bread containing hydrogenated poly-1-decene at a concentration of 500mg/kg could be consumed by a 60-kg person before the ADI of 0–6mg/kg of body weight was exceeded. However, it was considered highly unlikely that a person would consume this amount of bread containing hydrogenated poly-1-decene at the maximum level of use each day.

An addendum to the toxicological monograph was prepared.

3.1.6 **Preservative**

3.1.6.1 *Natamycin (pimaricin)*

Natamycin (pimaricin) is a polyene macrolide antibiotic produced by submerged aerobic fermentation of *Streptomyces natalensis* and related species. The fermentation process takes several days, after which the antibiotic is isolated by extraction from broth or by extraction of the mycelium.

Natamycin is used as a food additive to control the growth of yeasts and moulds on the surface of cheese and other non-sterile products, such as meat and sausages.

Natamycin was evaluated by the Committee at its twelfth and twentieth meetings (Annex 1, references 17 and 41). At its twentieth meeting, the Committee established an ADI of 0–0.3mg/kg of body weight. The present evaluation was conducted in response to a request by the Codex Committee on Food Additives and Contaminants at its Thirty-second Session (4).

The Committee considered information on the current uses of natamycin, data on its intake and biological data that had not been evaluated previously.

Uses. Because natamycin is active against yeasts and moulds, but not bacteria, it is used in foods that undergo a ripening period after processing. Its low solubility in water and most organic solvents makes it appropriate for the surface treatment of foods.

Natamycin is used topically in veterinary medicine to treat mycotic infections, such as ringworm in cattle and horses. Previously, it was used topically against fungal infections of the skin and mucous membranes in humans. Its medical use is now confined to topical treatment of fungal infections of the cornea and the prevention of such infections in contact lens wearers.

Assessment of intake. The Committee noted that as the draft Codex General Standard for Food Additives proposes restricted use of natamycin only in cheese and in dried, non-heat-treated meats, intake would not be expected to exceed the ADI.

Data submitted by Australia, Germany, New Zealand, the United Kingdom and the USA indicated that the intakes at mean and high percentiles of consumption were well below the ADI, although the estimates for the United Kingdom and the USA covered cheese consumption only. The estimated mean intakes by consumers ranged from 0.01 to 0.03 mg/kg of body weight per day (representing 3% and 9% of the ADI in Germany and the United Kingdom, respectively), and those by consumers at high percentiles were 0.03–0.08 mg/kg of body weight per day (representing 9% and 27% of the ADI in Australia and the United Kingdom, respectively), if it is assumed that natamycin was used at 40 mg/kg in all cheese products and 20 mg/kg in all cured meat products, as proposed in the draft Codex General Standard for Food Additives. The estimated intakes of natamycin were lower when national levels of use were assumed.

Toxicological studies. The Committee considered eight studies that had not been evaluated previously and had been conducted before the 1980s. A study of single intraperitoneal administration was considered to be irrelevant to the safety assessment of an ingested substance. The results of two studies of genotoxicity in three bacterial systems (*Bacillus subtilis*, *Salmonella typhimurium* and *Escherichia coli*) were negative.

Two studies in rats and one in dogs given radiolabelled material for investigation of the distribution and elimination of the compound supported the previous conclusion that natamycin is excreted primarily in the faeces, with minimal absorption. The only adverse effect reported in a short-term study of toxicity in dogs was diarrhoea, which occurred most frequently in animals given the highest dose (equivalent to 25 mg/kg of body weight per day); however, the usefulness of this study was limited, as only two dogs were tested.

In a study of developmental toxicity, an aqueous suspension of natamycin at 500 mg/l was given to groups of 20–26 rabbits at a dose of 0, 5, 15 or 50 mg/kg of body weight per day by gavage on days 6–18 of gestation. The maternal mortality rate was 0%, 5%, 9% and 19% at the four doses, respectively. No clinical signs of toxicity were observed in the does, and the cause of death was unknown. The mean maternal body weight, pregnancy rate, number of implantation sites, number of resorption sites, numbers of live and dead fetuses, proportion of viable fetuses and incidence of soft-tissue anomalies were comparable in the treated groups and a control group given the vehicle only. The fetal body weight in the group dosed at 15 mg/kg of body weight by gavage was lower than that of fetuses in the control group given the vehicle only. The incidence of extra sternbrae was increased at the two highest doses in comparison with the control

group, but not in a dose-related manner. However, in view of the unusual sensitivity of the gastrointestinal tract of rabbits to poorly absorbed substances and to compounds with antimicrobial activity, this study was considered unsuitable for deriving the ADI.

Microbiological studies. The antifungal activities of natamycin and other polyenes depend on their binding to cell membrane sterols, primarily ergosterol, the principal sterol in fungal membranes. Oomycetes fungi and bacteria are insensitive to these antibiotics because their membranes lack ergosterol.

Use of natamycin as an antifungal agent in food may result in exposure of the indigenous microflora to trace quantities of antimicrobial residues. The human intestinal microflora is a complex mixture of more than 400 bacterial species, consisting primarily of bacterial cells at a concentration of approximately 10^{11} – 10^{12} colony-forming units per gram (CFU/g). Fungi are much less abundant than bacteria in the human gastrointestinal tract, the concentration of yeast in stool samples from healthy subjects being up to 10^5 CFU/g. As bacteria are not affected by polyenes, natamycin residues should not harm them; as yeasts are found in low quantities, the consequences of exposure to traces of natamycin would be minimal.

Several studies in experimental animals indicated a lack of antimicrobial activity in the colon, suggesting that natamycin was degraded into microbiologically inactive compounds by bacterial flora. However, no data were available on the degradation of natamycin by human intestinal microflora. In one study, natamycin was present in faecal specimens of volunteers who ingested 500 mg of the compound, indicating that it is incompletely absorbed or degraded.

As emergence of resistance to antimicrobials is a concern, the Committee evaluated the possible development of resistance among microflora as a consequence of exposure to natamycin. A preparation containing 50% natamycin has been used since the 1980s to preserve cheese and sausages. Surveys in cheese warehouses and in dry-sausage factories where the preparation has been used showed no change in the composition or the sensitivity of the contaminating fungal flora. All but one of the species of yeasts and moulds isolated in cheese warehouses where natamycin was used were inhibited by similarly low concentrations (0.5–8 µg/ml). In another study, 26 strains of fungi were isolated in eight warehouses where natamycin was used and two warehouses where it had never been used, and were tested for sensitivity to the compound; no insensitive yeasts or moulds were found. The results of laboratory experiments to induce resistance to natamycin in strains of fungi isolated from cheese

warehouses indicated that, after 25–30 transfers to media with increasing concentrations of natamycin, none of the strains had become less sensitive. When the sensitivity of yeasts and moulds isolated from dry-sausage factories where natamycin had been used for several years was compared with that of isolates from factories where natamycin had never been used, no significant differences were demonstrated.

It has been found difficult to induce resistance to polyenes, especially natamycin, in fungi in vitro. Resistant isolates invariably show reduced metabolic and growth rates and, in the absence of polyenes, readily revert to normal metabolism, growth and sensitivity to natamycin. One means of obtaining isolates resistant to natamycin is successive subculturing in vitro in the presence of gradually increasing concentrations of the polyene. Typically, such isolates are resistant only up to the highest concentration to which they have been exposed. After 25 passages, the concentration that inhibited *Candida albicans* was minimally increased, from 2.5–12 µg/ml to 12–50 µg/ml.

Evaluation. Natamycin is a polyene macrolide antibiotic that is effective against yeasts and moulds but not against bacteria or oomycetes fungi. The antifungal activities of natamycin depend on its binding to cell membrane sterols, primarily ergosterol, the principal sterol in fungal membranes, which is absent in bacteria. The use of natamycin as an antifungal agent in food may result in exposure of the indigenous flora to trace quantities of antimicrobial residues. As bacteria in the human gastrointestinal tract are not affected by polyenes, the Committee concluded that natamycin would not have an effect and that disruption of the barrier to colonization of the intestinal tract was therefore not a concern. Fungi are much less prevalent than bacteria in the human gastrointestinal tract, and, in light of the negative results of the studies of acquired resistance, selection of natamycin-resistant fungi was not considered an issue.

The Committee noted the finding of extra sternebrae in the study of developmental toxicity in rabbits, in which a dose-related increase in the mortality rate was also reported. It considered, however, that administration of an antimicrobial agent to rabbits by gavage was an inappropriate way of testing for developmental toxicity. In addition, extra sternebrae have been described as a skeletal variation rather than a frank sign of teratogenicity. Thus, the Committee did not consider the finding of extra sternebrae to be evidence that natamycin is teratogenic.

The Committee confirmed the previously established ADI of 0–0.3 mg/kg of body weight for natamycin, which was based on observations of gastrointestinal effects in humans. The Committee noted that the estimated intakes of natamycin, based on maximum levels of use

in cheese and processed meats proposed in the draft Codex General Standard for Food Additives, do not exceed this ADI.

A toxicological monograph was prepared and the existing specifications were revised. The title of the specifications was changed from pimaricin to natamycin, the commonly used designation. The specifications were made tentative, pending the receipt of information on the level and determination of water content, limit for lead, specific rotation, assay value and method of assay for the commercial product. This information was required for evaluation in 2003.

3.1.7 **Sweetening agent**

3.1.7.1 *D-Tagatose*

D-Tagatose is a keto-hexose, an epimer of D-fructose inverted at C-4, with a sweet taste. It is obtained from D-galactose by isomerization under alkaline conditions in the presence of calcium.

D-Tagatose was evaluated by the Committee at its fifty-fifth meeting (Annex 1, reference 149), when it concluded that the available data indicated that D-tagatose is not genotoxic, embryotoxic or teratogenic. It noted that the increased liver weights and hepatocellular hypertrophy seen in Sprague-Dawley rats occurred concurrently with increased glycogen deposition; however, the reversal of increased glycogen storage after removal of D-tagatose from the feed was more rapid than regression of the liver hypertrophy. Although the gastrointestinal symptoms seen in adult humans with the expected daily intake of D-tagatose were minor, the Committee was concerned about the increased serum uric acid concentrations observed in a number of studies in humans after administration of either single or repeated doses of D-tagatose. Similar increases were observed with other sugars, such as fructose, but D-tagatose appeared to be a more potent inducer of this effect. The Committee also noted that this effect of D-tagatose had not been studied in persons prone to high serum uric acid concentrations. The Committee concluded that an ADI could not be allocated for D-tagatose because of concern about its potential to induce glycogen deposition in the liver and liver hypertrophy and to increase the serum concentration of uric acid.

Two studies of up to 7 days' duration in Wistar and Sprague-Dawley rats given repeated doses of D-tagatose were submitted to the Committee at its fifty-fifth meeting, but the reports were received only in draft form and were not suitable for consideration at that time. The Committee therefore asked for the final reports and for further data to clarify the extent, mechanism and toxicological consequences of the increased serum uric acid concentrations observed in humans exposed to D-tagatose. At its present meeting, the Committee reviewed the

reports of the two studies in rats, the results of a study in volunteers (on the relevance of the glycogen deposition and liver hypertrophy) and some published studies on the increased uric acid concentrations in serum after intake of D-tagatose, other sugars and other food components.

Biological data. Review of the results of the studies considered by the Committee at its fifty-fifth meeting and comparisons with the data reviewed at the present meeting revealed a difference in sensitivity between Wistar and Sprague-Dawley rats. Sprague-Dawley rats given D-tagatose at a concentration of 50 g/kg of diet for 28 days showed increased hepatic glycogen only when they had not been fasted the night before necropsy, and this effect was not associated with any microscopic changes in the liver. In a 90-day study in which Sprague-Dawley rats were killed after fasting overnight, administration of D-tagatose at a concentration of 50 g/kg of diet had no adverse effect on the liver. In a 6-month study in Wistar rats in which the animals were killed after fasting 3, 7, 14 and 28 days and 3 and 5 months after treatment, administration of D-tagatose at concentrations of up to 100 g/kg of diet had no adverse effects. Wistar rats are therefore less susceptible to the hepatic effects of D-tagatose than Sprague-Dawley rats. As D-tagatose stimulated glycogen deposition to a similar degree in the two rat strains in short-term studies, the difference is likely to occur at a later stage, during glycogen-induced or other stimulation of liver growth.

The authors suggested that the increase in normal liver mass seen in fasted rats fed diets containing 100 or 200 g/kg D-tagatose is triggered by increased postprandial storage of liver glycogen resulting from simultaneous feeding of D-tagatose and glucose equivalents. In order to test this hypothesis, the effects of separate and simultaneous administration of D-tagatose and glycogen precursors on liver weight and glycogen level were investigated in Wistar and Sprague-Dawley rats. The results neither supported nor invalidated the hypothesis.

As several studies have been performed in healthy volunteers and in patients with diabetes, the number of persons varying from 4 to 73, the Committee based its toxicological evaluation on the data from these studies. The length of these studies varied from several days to several weeks; one study of 12 months' duration included only a limited number of patients with type 2 diabetes. The toxicological aspects investigated included gastrointestinal effects, increased serum uric acid concentrations and hepatic effects.

Mild gastrointestinal symptoms were reported in only one study, in 3 of 10 patients with type 2 diabetes receiving D-tagatose at 10 g/day for several days, whereas in other studies diarrhoea was observed only in patients receiving 25 g three times daily for 8 weeks. In healthy individuals, administration of a single dose of 30 g induced diarrhoea in

some persons only, whereas other studies showed no laxative effect of single doses of D-tagatose as high as 75 g.

The serum or plasma concentration of uric acid was increased transiently in some studies, but the increased uric acid concentration was above the normal range for a number of days in only one study of persons receiving 75 g/day. The other studies showed either no increase or a transient increase in serum uric acid concentrations within the normal range.

In a 28-day study in which 15 g of D-tagatose or 15 g of sucrose were given three times daily to volunteers, magnetic resonance imaging was used to determine liver volume, and glycogen concentrations and several clinical chemical parameters were measured. The results did not reveal any relevant effect on the liver. In addition, no diarrhoea and no increase in serum uric acid concentration were observed. Therefore, the NOEL was 45 g/person per day, equivalent to 0.75 g/kg of body weight per day (for a person weighing 60 kg).

Evaluation. The Committee considered the 28-day study in which humans received a daily dose of 45 g of D-tagatose or sucrose in three divided doses as most representative of human dietary intake and therefore most relevant for assessing the acceptable intake of D-tagatose accurately. While effects were observed after administration of a single dose of 75 g, no effects were seen following administration of three daily doses of 15 g of D-tagatose, equivalent to 0.75 g/kg of body weight per day. The Committee established an ADI of 0–80 mg/kg of body weight on the basis of this NOEL and a safety factor of 10.

Assessment of intake. D-Tagatose is proposed for use as a bulk sweetener in low-energy foods, such as edible ices (at a concentration of 3 g/kg), chewing-gum and confectionery (at 15 g/kg), breakfast cereals (at 15 g/kg) and soft drinks (at 1 g/kg). At its present meeting, the Committee considered that the predicted intakes of D-tagatose determined at the fifty-fifth meeting, which were based on the manufacturers' proposed levels of use and individual dietary records in several countries, were conservative. This was because use had been assumed in the entire food category rather than only in the low-energy food component. The mean consumer intakes of D-tagatose from all proposed uses (except chewing-gum, dietary supplements and meal replacements) predicted for Australia, the Member States of the European Union and the USA ranged from 3 to 9 g/day (63–190% of the ADI), and the predicted intakes by persons at high percentiles of consumption were up to 18 g/day (375% of the ADI). On the basis of the information on possible uses, the Committee concluded that the ADI for D-tagatose may be exceeded by some groups of the population.

A toxicological monograph was prepared. The specifications prepared by the Committee at its fifty-fifth meeting were maintained.

3.1.8 **Thickening agents**

3.1.8.1 *Carrageenan and processed Eucheuma seaweed*

Carrageenan, a substance with hydrocolloid properties owing to the presence of sulfated polyglycans with average relative molecular masses well above 100000, is derived from a number of seaweeds of the family Rhodophyceae. It has no nutritional value and is used in food preparation for its gelling, thickening and emulsifying properties. Three main types of carrageenan, known as ι -, κ - and λ -carrageenan, are used commercially in the food industry. These names do not reflect definitive chemical structures but only general differences in the composition and degree of sulfation at specific locations in the polymer. Processed *Eucheuma* seaweed is derived from either *E. cottonii* (κ -carrageenan) or *E. spinosum* (λ -carrageenan), which are also Rhodophyceae.

Carrageenan is obtained by extraction of the seaweed into water or aqueous dilute alkali and may be recovered by precipitation with alcohol, by drying in a rotary drum or by precipitation with aqueous potassium chloride and subsequent freezing. In contrast, processed *Eucheuma* seaweed is prepared by soaking the cleaned seaweed in alkaline solution for a short time at elevated temperatures. The treated material is then thoroughly washed with water to remove residual salts and further washed with alcohol, dried and milled to a powder. For both carrageenan and processed *Eucheuma* seaweed, the alcohols that may be used during purification are restricted to methanol, ethanol and isopropanol. The articles of commerce may contain sugars added for standardization purposes, salts to obtain specific gelling or thickening characteristics, or emulsifiers carried over from the drum-drying process.

Carrageenan was reviewed by the Committee at its thirteenth, seventeenth, twenty-eighth and fifty-first meetings (Annex 1, references 19, 32, 66 and 137). At its twenty-eighth meeting, the Committee established an ADI “not specified”¹ on the basis of the results of a number

¹ ADI “not specified” is used to refer to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effect and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for reasons stated in the individual evaluation, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice, i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.

of toxicological studies on carrageenans obtained from various sources.

Processed *Eucheuma* seaweed was considered by the Committee at its thirtieth, thirty-ninth, forty-first, forty-fourth and fifty-first meetings (Annex 1, references 73, 101, 107, 116 and 137). At its forty-fourth meeting, the Committee concluded that, because of the chemical relationship between processed *Eucheuma* seaweed and traditionally refined carrageenan, the toxicological data on carrageenan were relevant to the safety assessment of the carrageenan polysaccharide constituents of processed *Eucheuma* seaweed, but could not replace adequate toxicological studies on processed *Eucheuma* seaweed itself. At its fifty-first meeting, the Committee reviewed the results of a 90-day study on toxicity in rats fed processed *Eucheuma* seaweed from *E. cottonii* and *E. spinosum*. The Committee concluded that the toxicity of this material was sufficiently similar to that of carrageenan to allow extension of the previous ADI “not specified” for carrageenan to a group ADI that covered processed *Eucheuma* seaweed. The Committee also considered all studies on carrageenan that had been published since its twenty-eighth meeting and, for the earlier studies, noted the identity of the source material and the type of carrageenan, when these could be identified. It expressed concern about the potential promotion of colon carcinogenesis by carrageenans and processed *Eucheuma* seaweed and therefore made the group ADI “not specified” temporary, pending clarification of the significance of the promotion of colon cancer observed in studies in rats. At its present meeting, the Committee reviewed the available evidence for the tumour-promoting and related effects of these compounds in rat colon.

Assessment of intake. Carrageenan and processed *Eucheuma* seaweed are used as thickeners, gelling agents, stabilizers or emulsifiers in a wide range of foods at concentrations of up to 1500mg/kg. Per capita intakes in 1995 derived from “poundage” (disappearance) data in Europe and the USA ranged from 28 to 51 mg/day. These estimates corresponded to those reported for 1993 by the Seaweed Industry Association of the Philippines on the basis of sales of 44mg/person per day for the populations of Canada and the USA and 33 mg/person per day for European populations.

The estimates derived from poundage data were also consistent with those derived for the population of the USA from model diets, with reported mean intakes of carrageenan of 20mg/day for all consumers and 40mg/day for persons at the 90th percentile of consumption (derived by multiplying the mean by a factor of 2). The intakes were derived from data on the food consumption of individuals aged

2 years and over that were available in 1976 from nutrition surveys in the USA, combined with the results of a 2-week study by the Marketing Research Corporation of America on the frequency of food consumption.

Biological data. Two studies showed that carrageenan administered before, during and after administration of known carcinogens (dimethylhydrazine, azoxymethane, *N*-methyl-*N*-nitrosourea) enhanced the tumorigenicity of these carcinogens. One of the studies involved administration of carrageenan at 150 g/kg of diet, which resulted in decreased body-weight gain. In the second study, involving administration of carrageenan at 60 g/kg of diet, the body-weight gain of treated animals was comparable to that of controls. The increased incidence of tumours seen under these circumstances may have resulted from promotion but may also have resulted from altered toxicokinetics or biotransformation of the carcinogen. In addition, there were indications that the bacterial flora had been altered as a result of administration of carrageenan. In a separate study conducted according to a classical tumour initiation–promotion protocol, in which rats were given dimethylhydrazine, subsequent administration of carrageenan at dietary concentrations of up to 50 g/kg did not result in a statistically significant increase in the incidence of colon tumours over that seen with dimethylhydrazine alone.

Two further studies in rats involved use of a conventional tumour initiation–promotion protocol but in which formation of aberrant crypt foci was the end-point, instead of tumour formation. Rats were given azoxymethane with or without subsequent administration of carrageenan in their drinking-water. The higher concentration of carrageenan, 25 g/kg, was given in the form of a solid gel, which may have altered the food and water consumption patterns of the animals. The first study demonstrated that dietary administration of carrageenan after the carcinogen decreased the number of aberrant crypt foci seen relative to the number observed with the carcinogen alone, but significantly increased their size. A subsequent study in rats injected with human faecal microflora showed no effect of carrageenan on either the number or size of aberrant crypt foci. As the relationship between aberrant crypt foci and tumorigenesis is still unclear, it is difficult to interpret the biological significance of these results.

Increased cell proliferation has frequently been postulated as a mechanism of non-genotoxic carcinogenicity or tumour promotion. The preferred methods of assessing cell proliferation are based on histological techniques, which allow identification of the nature and location of proliferating cells. There was no consistent pattern of colon damage in rats treated with carrageenan for prolonged periods.

Some studies showed caecal enlargement, but most did not show histological damage. In one study in which rats underwent autoradiographic examination, no significant difference from controls in the number of cells per crypt or in the proportion of labelled cells was seen in rats fed a diet containing carrageenan at 74g/kg for 28 days.

Methods for measuring cell proliferation that are based on measurement of cell cycle-dependent enzyme activities, such as thymidine kinase activity, are cruder means of measuring overall cell proliferation in an entire tissue specimen. A significant increase in thymidine kinase activity, expressed relative to protein content, was found in homogenized mucosal scrapings from the colon of rats fed diets containing carrageenan at 26 or 50g/kg for 4 weeks; no significant effects were observed in the animals fed 0, 6.5 or 13g/kg carrageenan in the diet for 4 weeks. Histological examination revealed no evidence of infiltration by inflammatory cells in any of the treated groups. In another study, the increased thymidine kinase activity observed in rats fed diets containing carrageenan at 50g/kg returned to the basal level within 28 days when the animals were returned to a diet with no carrageenan. No increase in thymidine kinase activity was seen in animals receiving diets containing 2 or 15g/kg carrageenan for 28 days. Staining for proliferating cell nuclear antigen (PCNA) revealed a significant increase in PCNA-positive cells in the upper third of the crypts of rats receiving a diet containing carrageenan at 50g/kg for 91 days, but not after 28 or 64 days followed by a 28-day recovery period on a normal diet. No PCNA-positive cells were observed at the luminal surface. The pattern of staining for PCNA seen with carrageenan was considered indicative of an adaptive response, which would not contribute to an increased risk for colonic neoplasia.

In one study, carrageenan inhibited gap-junctional intercellular communication *in vitro*. However, the mechanism of action was different from that of a known tumour-promoting agent, phorbol ester, and the relevance of this observation is unclear for a substance that is not absorbed *in vivo*.

Evaluation. In a recent study with a classical tumour initiation-promotion protocol, administration of carrageenan at concentrations of up to 50 g/kg of diet did not promote colon carcinogenesis in rats given dimethylhydrazine. The Committee noted, however, that, in two studies that showed enhancement of colon carcinogenesis in rats, higher dietary concentrations of carrageenan were used and carrageenan was administered before, during and after the carcinogens. The enhanced carcinogenicity seen under these circumstances may have resulted from promotion or from altered toxicokinetics or bio-transformation of the carcinogen. Therefore, the mechanism of the

enhancement of colon carcinogenesis in these studies remains unresolved. Continuous feeding of high doses of carrageenan caused a generalized proliferative response, measured as increased thymidine kinase activity, in the mucosal tissue of the colon of male rats. This effect might play a role in the observed enhancement of the tumorigenicity of known colon carcinogens by high dietary concentrations of carrageenan. However, a proliferative effect of carrageenan on the mucosa of the colon was seen only at a dietary concentration of 26 g/kg or more. No effect was seen at a concentration of 15 g/kg in the diet, corresponding to 750 mg/kg of body weight per day, which greatly exceeded the estimated human intake of carrageenan and processed *Eucheuma* seaweed of 30–50 g/person per day from their use as food additives. Bearing in mind that the enhancement of colon carcinogenesis in rats was seen at much higher concentrations and that carrageenan at 50 g/kg of diet did not promote tumours in rat colon in a classical initiation–promotion study, the Committee considered that the intake of carrageenan and processed *Eucheuma* seaweed from their use as food additives was of no concern. It therefore allocated a group ADI “not specified”¹ to the sum of carrageenan and processed *Eucheuma* seaweed.

An addendum to the toxicological monograph was prepared. The existing specifications for both carrageenan and processed *Eucheuma* seaweed were revised by incorporating more complete descriptions of the analytical procedures for the determination of lead, cadmium and mercury and by raising the acceptable limit for lead from 2 mg/kg to 5 mg/kg and the acceptable limit for cadmium from 1 mg/kg to 2 mg/kg. These limits were raised to take into account new information on inadequacies of the analytical methods for determination of these elements, which are due to the high salt content of the polysaccharides of both processed *Eucheuma* seaweed and carrageenan. The changes were not made because of information about higher concentrations of lead and cadmium than those previously considered by the Committee. The Committee also observed that the new limits are consistent with the limits established for these heavy metals in specifications for other hydrocolloids, such as alginic acid.

3.1.8.2 Curdlan

Curdlan (synonym, β -1,3-glucan) is a linear polymer of high relative molecular mass, consisting of β -1,3-linked glucose units. Curdlan is produced by fermentation of pure cultures from a non-pathogenic, non-toxinogenic strain of *Agrobacterium* Biovar1 (identified as *Alcaligenes faecalis* var. *myxogenes* at the time of its isolation) or

¹ See footnote on page 32.

Alcaligenes radiobacter. Curdlan is recovered from the fermentation medium by addition of acid and alkali to disrupt the cells, which releases the curdlan into the medium, followed by separation by centrifugation. It is then washed with copious amounts of water to eliminate mineral salts and other water-soluble substances that may have been carried over from the fermentation broth. The commercial product is an odourless or nearly odourless, tasteless, white to nearly-white spray-dried powder.

The use of curdlan in a wide variety of foods is based on its ability to form an elastic gel upon heating in an aqueous suspension. Thus, it can be used in processed meat, fish and poultry products and in gelatins, puddings and fillings as a firming or gelling agent or as a stabilizer or thickener.

The Committee reviewed curdlan at its fifty-third meeting (Annex 1, reference 143), when it allocated a temporary ADI “not specified”,¹ pending information on the use of curdlan, including the maximum and typical expected levels in the food categories in which it is proposed for use in the draft Codex General Standard for Food Additives, and on the consumption in various regions of the world of foods that might contain curdlan.

Use of curdlan is based on its physical properties, which imply a self-limiting level of use in solid foods. A submission from the USA described a model constructed to predict the intakes of curdlan by a long-term consumer on the basis of a study of the frequency of consumption of foods in 1982–1988 from the Market Research Corporation of America, and average portion sizes from a 3-day national food consumption survey conducted in 1987–1988 by the United States Department of Agriculture. Intake was assessed on the basis of the self-limiting levels of use (20 mg/kg of processed meat, 15 mg/kg of processed poultry and fish, 10 mg/kg of dairy products, 35 mg/kg of egg products, 15 mg/kg of grain products and pasta, 30 mg/kg of cereals and starch desserts, 20 mg/kg of gravies and sauces and 40 mg/kg of gelatins). The resulting mean intake by consumers was estimated to be 3.6 g/person per day, corresponding to 60 mg/kg of body weight per day.

The sponsor submitted an estimate based on daily food intake per capita and typical levels of use in Japan (15mg/kg of processed meat, 10mg/kg of processed poultry and fish, 5mg/kg of dairy products, 30mg/kg of egg products, 10mg/kg of grain products and pasta, 10mg/kg of cereals and starch desserts, 10mg/kg of gravies and sauces and 30mg/kg of gelatins). The mean intakes were estimated to be

¹ See footnote on page 32.

0.77 g/person per day, corresponding to 13 mg/kg of body weight per day, for typical levels of use and 1.7 g/person per day, corresponding to 28 mg/kg of body weight per day, for maximum levels of use.

Estimates of the intake of curdlan based on individual dietary records were submitted by the USA on the basis of a survey by the Department of Agriculture and the Continuing Survey of Food Intakes by Individuals (1989–1992). When intake was estimated on the basis of the upper limit of the range of recommended use, the intake of curdlan by consumers was 20 mg/kg of body weight per day for consumption at the mean and 47 mg/kg of body weight per day for consumption at the 90th percentile. When intake was estimated on the basis of self-limiting levels of use, the intake of consumers was 30 mg/kg of body weight per day for consumption at the mean and 68 mg/kg of body weight per day for consumption at the 90th percentile.

The data on uses and intake requested by the Committee at its fifty-third meeting were provided and raised no safety concern. The Committee therefore established an ADI “not specified”¹ for use of curdlan as a food additive.

The existing specifications were revised, with minor changes.

3.1.9 *Miscellaneous substances*

3.1.9.1 *Acetylated oxidized starch*

Acetylated oxidized starch is a chemically modified root or grain starch. It is produced by oxidation of a slurry of starch granules in alkaline hypochlorite at low temperatures (21–38°C). The alkaline medium is neutralized with sodium bisulfite, and the resulting organic salts are removed by washing with water. The oxidized starch is then esterified with acetic anhydride under mildly alkaline conditions. The product is neutralized with hydrochloric acid, washed and dried.

Acetylated oxidized starch had not been evaluated previously by the Committee. At the present meeting, it was proposed for use as a binding agent in soft confectionery at a concentration of about 300 mg/kg — it is mixed with water, sugars and flavours in a batch process until a clear solution with a dry-solid content of 70% is obtained. The characteristics of the end-product important for confectionery use are gel strength and clarity. Acid hydrolysis results in starch products that are relatively unclear, and oxidized starch products result in overly soft confectionery. Acetylation of oxidized starch enhances the desired properties, resulting in a gummy, clear jelly. It

¹ See footnote on page 32.

can be used as a substitute for gelatin or gum arabic and would replace a large amount of sugar.

Acetylated oxidized starch has a stable configuration under normal conditions in food. It is hydrolysed slowly in the presence of strong acids, yielding glucose, gluconic acid and acetic acid. No degradation products are expected or known to result from storage or use of this substance in the preparation of foods at neutral pH. The substance is not known to sequester minerals, nor does it interact with proteins or vitamins. It has no known effect on other nutrients.

In a 14-day range-finding study in rats, administration of a diet containing acetylated oxidized starch at a concentration of 300 or 500 mg/kg increased the weights of full and empty caeca, and dilated caeca were found at autopsy. At the higher concentration, soft faeces also occurred. The NOEL was 100 mg/kg of diet.

In a 90-day study in rats given a diet containing acetylated oxidized starch, increased full and empty caecal weights were seen at the highest concentration of 300 mg/kg of diet. Macroscopic examination showed a dilated caecum in one male rat. Histological examination did not reveal changes in the caecal wall or other parts of the digestive tract. Increased caecal weights are a known response to high dietary concentrations of poorly digested carbohydrates in rats, due perhaps to an increased osmotic load of short-chain fatty acids produced by microbial degradation and the associated water retention. Focal hyperplasia of the urinary bladder epithelium was seen in 4 out of 10 male rats that received the highest dietary concentration but not in males given lower concentrations, in controls or in females. The change was probably treatment-related and a consequence of irritation of the urinary bladder by calculi. The NOEL was 100 mg/kg of diet, equivalent to 5900 mg/kg of body weight per day.

If acetylated oxidized starch was to be used only in jelly confectionery at a concentration of 300 g/kg and if the maximum consumption by consumers was 200 g of jelly confectionery per day, the maximum intake of acetylated oxidized starch would be 60 g/day.

The effects seen in the 14-day and 90-day studies in rats were similar to those observed with high dietary concentrations of other slowly digested carbohydrates and are commonly seen in rats given other modified starches in the diet. Because of the nature of acetylated oxidized starch and its similarity to other modified starches with non-systemic effects, the Committee established an ADI “not specified”,¹ on the basis of the known uses of acetylated oxidized starch as an ingredient in confectionery products.

¹ See footnote on page 32.

A toxicological monograph was prepared. New specifications for acetylated oxidized starch were prepared and incorporated into the specifications for modified starches.

3.1.9.2 *α-Cyclodextrin*

α-Cyclodextrin is a non-reducing cyclic saccharide composed of six glucose units linked by α -1,4 bonds. It is produced by the action of cyclodextrin glucosyltransferase (CGTase, EC 2.4.1.19) on hydrolysed starch syrups at neutral pH (6.0–7.0) and moderate temperatures (35–40°C). The annular structure of *α*-cyclodextrin provides a hydrophobic cavity that allows formation of inclusion complexes with a variety of non-polar organic molecules of appropriate size. The hydrophilic nature of the outer surface of the cyclic structure makes *α*-cyclodextrin water-soluble.

The principal method for the isolation and purification of *α*-cyclodextrin takes advantage of its complex-forming ability. At the end of the reaction, 1-decanol is added to the reaction mixture to form an insoluble 1:1 inclusion complex of *α*-cyclodextrin:1-decanol. The complex is continuously mixed with water and separated from the reaction mixture by centrifugation. The recovered complex is resuspended in water and dissolved by heating. Subsequent cooling leads to precipitation of the complex. The precipitate is recovered by centrifugation, and 1-decanol is removed by steam distillation. Upon cooling, *α*-cyclodextrin crystallizes from the solution. The crystals are removed by filtration and dried, yielding a white crystalline powder with a water content of less than 11%. The purity on a dried basis is at least 98%.

The hydrophobic cavity and the hydrophilic outer surface of *α*-cyclodextrin form the basis for its use in the food industry. *α*-Cyclodextrin, like its homologues β - and γ -cyclodextrin, can function as a carrier and stabilizer for flavours, colours and sweeteners; as an absorbent for suppression of undesirable flavours and odours in foods; as an absorbent for suppression of halitosis (in breath-freshening preparations); and as a water-solubilizer for fatty acids and vitamins.

α-Cyclodextrin had not been evaluated previously by the Committee, but the structurally related compound β -cyclodextrin was evaluated at the forty-first and forty-fourth meetings (Annex 1, references 107 and 116), and γ -cyclodextrin was evaluated at the fifty-first and fifty-third meetings (Annex 1, references 137 and 143). At its present meeting, the Committee noted the close structural similarity between *α*- and β -cyclodextrin (seven glucose units) and γ -cyclodextrin (eight glucose units), which permitted comparisons of the metabolism and toxicity of these compounds.

Biological data. α -Cyclodextrin, like β -cyclodextrin, is not digested in the gastrointestinal tract but is fermented by the intestinal microflora. In germ-free rats, α -cyclodextrin is almost completely excreted in the faeces, whereas γ -cyclodextrin is readily digested to glucose by the luminal and/or epithelial enzymes of the gastrointestinal tract. At low concentrations in the diet (about 20 g/kg), α -cyclodextrin is absorbed intact from the small intestine and is then excreted rapidly in the urine. The majority of the absorption takes place after metabolism of the substance by the microflora in the caecum. Although no studies of metabolism in humans in vivo were available, in vitro studies indicated that α - and β -cyclodextrin, unlike γ -cyclodextrin, cannot be hydrolysed by human salivary and pancreatic amylases.

The acute toxicity of α -cyclodextrin was studied in mice and rats that received the substance by intraperitoneal or intravenous injection. It caused osmotic nephrosis, probably because it was not degraded by lysosomal amylases. At high doses, this led to renal failure.

The results of short-term (28-day and 90-day) studies of the toxicity of α -cyclodextrin indicated that it had little effect when given orally to rats or dogs. After administration of a very high dietary concentration (200 g/kg), caecal enlargement and associated changes were seen in both species. This effect was probably the consequence of the presence of a high concentration of an osmotically active substance in the large intestine. No studies of intravenous administration were available to permit a comparison of the systemic toxicity of this compound with that of β - and γ -cyclodextrin.

Studies conducted in mice, rats and rabbits given α -cyclodextrin in the diet at concentrations of up to 200 g/kg did not indicate any teratogenic effects. Similarly, the results of assays for genotoxicity were negative. No long-term studies of toxicity, carcinogenicity or reproductive toxicity have been conducted with α -cyclodextrin, but the Committee concluded that, given the known fate of this compound in the gut, such studies were not required for an evaluation.

In vitro, α -cyclodextrin, like β -cyclodextrin, sequestered components of the membranes of erythrocytes, causing haemolysis. The threshold concentration for this effect was, however, higher than that observed with β -cyclodextrin.

While the potential interaction of α -cyclodextrin with lipophilic vitamins, which might impair their bioavailability, has not been studied directly, such an effect was considered unlikely, by analogy with the results of studies with β -cyclodextrin. Complexes between fat-soluble vitamins and β -cyclodextrin have been shown to have greater bioavailability than uncomplexed forms.

The enzyme cyclodextrin-glycosyltransferase, which is used in the production of α -cyclodextrin, is derived from a non-genotoxic, non-toxinogenic source and is completely removed from α -cyclodextrin during purification.

Assessment of intake. The predicted mean intake of α -cyclodextrin by consumers, based on individual dietary records for 1994–1998 in the USA and the proposed maximum levels of use in a variety of foods, would be 1.7 g/day (28 mg/kg of body weight per day) for the whole population and 1.6 g/day (87 mg/kg of body weight per day) for children aged 2–6 years. The main contributors to the total intake of α -cyclodextrin are likely to be soya milk and sweets. For persons at the 90th percentile of consumption, the predicted intake of α -cyclodextrin would be 3 g/day (50 mg/kg of body weight per day) for the whole population and 2.6 g/day (140 mg/kg of body weight per day) for children aged 2–6 years.

Evaluation. No studies of human tolerance to α -cyclodextrin were submitted to the Committee, despite the potentially high dietary intake. Nevertheless, the Committee was reassured by the relatively low toxicity of this compound in animals and the fact that it was less toxic than β -cyclodextrin, for which studies of human tolerance were available. Furthermore, the fact that it is fermented in the gastrointestinal tract in an analogous manner to β -cyclodextrin supported the conclusion that, as in laboratory animals, it would be fermented to innocuous metabolites before its absorption in the human gastrointestinal tract.

The Committee concluded that, on the basis of the available studies on α -cyclodextrin and studies on the related compounds β -cyclodextrin and γ -cyclodextrin, for which ADIs have been allocated, there was sufficient information to allocate an ADI “not specified”.¹ This ADI was based on the known current uses of α -cyclodextrin within good manufacturing practice as a carrier and stabilizer for flavours, colours and sweeteners; as a water-solubilizer for fatty acids and certain vitamins; as a flavour modifier in soya milk; and as an absorbent in confectionery.

A toxicological monograph and new specifications for α -cyclodextrin were prepared.

3.1.9.3 Sodium sulfate

Sodium sulfate was evaluated by the Committee at its fifty-third meeting (Annex 1, reference 143), when a temporary ADI “not specified”¹ was established. The ADI was made temporary because information was required on the functional effect and actual uses of

¹ See footnote on page 32.

sodium sulfate in food. This information was provided to the Committee at its fifty-fifth meeting (Annex 1, reference 149), and the “tentative” designation was removed from the specifications. At that time, the temporary ADI was not reconsidered.

Sodium sulfate is used as a colour adjuvant. Worldwide consumption from its use in food is approximately 100 tonnes per year.

At its present meeting, the Committee noted that the results of the few published studies conducted in experimental animals do not raise concern about the toxicity of sodium sulfate. Little is absorbed from the gut, and it is therefore used clinically as a laxative. The small amount absorbed remains in the extracellular fluid space and is rapidly excreted via the kidneys. Minor adverse effects have been reported in a small number of clinical trials and in case reports. All of the effects were seen with preparations containing sodium sulfate and may have resulted from other components of the preparations.

In the absence of evidence of toxicity and given the current uses of this substance, the Committee allocated an ADI “not specified”¹ for sodium sulfate.

A toxicological monograph was not prepared. The specifications prepared by the Committee at its fifty-fifth meeting were maintained.

3.2 Revision of specifications

3.2.1 Acesulfame K

Acesulfame K is prepared in a three-step process in which sulfamic acid and diketene are reacted to produce an adduct, which undergoes cyclization to the acid form of acesulfame. This product is neutralized with potassium hydroxide to form the potassium salt.

The specifications for acesulfame K were revised. In addition to editorial revisions, a new criterion for purity with regard to the pH value of the aqueous solution was introduced, and the limit for lead was lowered from 10mg/kg to 1 mg/kg.

3.2.2 Blackcurrant extract

Blackcurrant extract is obtained from blackcurrant pomace by aqueous extraction. The main colouring principles are four anthocyanins (cyanidin 3-rutinoside, delphinidin 3-rutinoside, cyanidin 3-glucoside and delphinidin 3-glucoside). Most of the extracted sugars are fermented to alcohol, and virtually all the alcohol is removed during concentration of the extract by vacuum evaporation. Sulfur dioxide is used during the extraction process, and residual sulfur dioxide may

¹ See footnote on page 32.

be present in the final product. The commercial products are concentrated liquids, pastes or spray-dried powders. Spray-dried powder may contain an added carrier such as maltodextrin or glucose syrup. At its present meeting, the Committee revised the specifications to include a chromatographic identification test which distinguishes blackcurrant extract from other anthocyanin colours and removed the “tentative” designation.

3.2.3 *L-Malic acid*

The Committee received no information about the uses of L-malic acid, other than its well-established use as a flavouring agent. As DL- and L-malic acid are different compounds made by different manufacturing processes, the specifications for DL-malic acid were corrected by removing the reference to the specifications for L-malic acid.

3.2.4 *Oxystearin*

The specifications for oxystearin were considered by the Committee at its fifty-fifth meeting (Annex 1, reference 149). At that meeting, the Committee maintained the “tentative” designation, with the stipulation that the specifications would be withdrawn if information on the levels of, and a suitable analytical method for, epoxides was not provided by 1 May 2001. The Committee noted that oxystearin was no longer in commercial use as a food additive.

At its present meeting, the Committee withdrew the specifications, as the requested information had not been received. The Committee also withdrew the ADI of 0–25 mg/kg of body weight for oxystearin established at its seventeenth meeting (Annex 1, reference 32), as it considered that there could not be an ADI for a substance for which there were no specifications.

3.2.5 *Pectins*

Pectins consist mainly of the partially methylated esters of polygalacturonic acid and its ammonium, sodium, potassium and calcium salts. Amidated pectins also contain amides of polygalacturonic acid. Pectins are obtained by extraction in an aqueous medium of an appropriate edible plant material, usually citrus fruits or apples. Amidated pectins are obtained by treating the extract with ammonia under alkaline conditions.

The specifications for pectins were revised. The four separate tests for the identification of pectins contained in the specifications prepared at the thirty-ninth meeting (Annex 1, reference 101) were replaced by a new test involving enzymatic degradation, which is specific for pectins, as the Committee had been informed that the previous tests were

not adequate for all commercial samples of pectins. The Committee was also informed that new separation techniques were used which could result in contamination of pectins with insoluble organic compounds. Therefore, a new criterion for purity, the percentage of “total insolubles”, was introduced. In addition, the limits for copper, zinc and arsenic were deleted, and the limit for lead was lowered from 10mg/kg to 5mg/kg.

3.2.6 **Smoke flavourings**

Smoke flavourings are complex mixtures of components of smoke obtained by subjecting untreated hardwoods to pyrolysis in a limited, controlled amount of air, dry distillation at 200–800°C or exposure to superheated steam at 300–500°C. The major flavouring principles are carboxylic acids, compounds with carbonyl groups and phenolic compounds.

During manufacture of smoke flavourings, hazardous constituents such as polycyclic aromatic hydrocarbons are removed by subjecting wood smoke to aqueous extraction or to distillation, condensation and separation for collection of the aqueous phase. The aqueous smoke fraction, containing water-soluble constituents, can be diluted with water or extracted with an edible vegetable oil to produce a smoke flavouring with a higher concentration of non-polar constituents, which may be further extracted with food-grade substances, such as propylene glycol or aqueous solutions of polysorbates.

The commercial products may also contain additives such as emulsifiers, antifoaming agents and gums. Smoke flavourings may also be prepared in dry form by the addition of carriers such as yeasts, flours, salt, phosphates, carbohydrates and anticaking agents.

The specifications for smoke flavourings were considered by the Committee at its fifty-fifth meeting (Annex 1, reference 149) and were maintained as “tentative”, pending the receipt of information on an alternative solvent to benzene for use in the analysis of the carbonyl content. At its present meeting, the Committee revised the existing tentative specifications and removed the “tentative” designation. The revised specifications apply only to water-soluble distillates of condensed wood smoke, to their aqueous, vegetable oil or polysorbate extracts and to concentrates of these products. They do not apply to products derived from the water-insoluble tars, to certain commercial products or to pyroligneous acid, a by-product of the manufacture of charcoal by carbonation of wood in the absence of air.

3.2.7 **Tagetes extract**

Tagetes extract is obtained by hexane extraction of dried petals of *Tagetes erecta* L., with subsequent removal of the solvent. The major

colouring principles are the xanthophyll lutein and its dipalmitate (helenien). Other hydroxy derivatives of carotenes may be present, together with other oxy derivatives, such as epoxides. The product may contain fats, oils and waxes that occur naturally in the plant material. The articles of commerce are usually further formulated, e.g. in order to standardize the colour content or to obtain water-soluble or dispersible products.

The specifications for tagetes extract were considered by the Committee at its fifty-fifth meeting (Annex 1, reference 149) and were designated as “tentative”, pending the receipt of information on the composition of the commercial products, a test for the identification of xanthophylls and a method of assay. As the Committee had received the requested information, it revised the existing tentative specifications and removed the “tentative” designation.

3.3 Revision of limits for metals in food additives

At its fifty-fifth meeting (Annex 1, reference 149), the Committee began to implement a systematic 5-year programme to replace the outdated test for heavy metals (as lead) in all existing food additive specifications with appropriate limits for individual metals of concern. Limits for lead and arsenic in 43 emulsifiers were proposed. As no alternative proposals were received by the Secretariat before the deadline for submission of data for the present meeting, the new published limits (Annex 1, reference 151) were adopted, replacing those published in the *Compendium of food additive specifications* and its addenda (Annex 1, references 103, 109, 118, 124, 133, 139, 145 and 151).

The second group of substances, considered at the present meeting, comprised 10 anticaking agents, 17 flavour enhancers, 10 sweetening agents and 13 thickening agents. In response to the call for data, proposed limits and data to support the proposals were received for sodium ferrocyanide. Comments and proposals only were received for calcium silicate, magnesium silicates (synthetic), silicon dioxide (amorphous), sodium aluminosilicate, monosodium L-glutamate, sorbitol, lactitol, xylitol, ammonium alginate, tara gum, methyl cellulose, ethyl cellulose, methylethyl cellulose, powdered cellulose, hydroxypropyl cellulose and hydroxypropylmethyl cellulose.

All the comments, proposals and supporting data were taken into account. Comments on the Committee’s new proposed limits (see Table 1) are invited. When higher limits are requested, analytical data in support of such limits must be provided. If alternative values and supporting data are not received by the deadline for submission of data for the fifty-ninth meeting of the Committee, the proposed limits will supersede the existing ones, replacing those published in the

Table 1

Limits for metals in food additives

Category	Additive name	INS No.	Limits (mg/kg)				
			Arsenic	Lead	Mercury	Cadmium	Other elements
Anticaking agents	Aluminium silicate	0559	—	5	—	—	
	Calcium aluminium silicate	0556	—	5	—	—	Iron <50
	Calcium silicate	0552	—	5	—	—	Iron <50
	Ferrocyanides of calcium, potassium and sodium	0538	3	5	—	—	Copper <10, zinc <25
	Magnesium oxide	0530	—	2	—	—	
	Magnesium silicates (synthetic)	0553a	—	5	—	—	Iron <10
	Silicon dioxide (amorphous)	0551	—	5	—	—	
	Sodium aluminosilicate	0554	—	5	—	—	
	Tricalcium phosphate	0341(iii)	—	4	—	—	Iron <50
	Trimagnesium phosphate	0342(iii)	—	4	—	—	Iron <5
	Calcium 5'-guanylate	0629	—	1			
	Calcium 5'-inosinate	0633	—	1			
Flavour enhancers	Calcium 5'-ribonucleotides	0634	—	1	—	—	
	Calcium di-L-glutamate	0623	—	1	—	—	
	Dipotassium 5'-guanylate	0628	—	1	—	—	
	Dipotassium 5'-inosinate	0632	—	1	—	—	
	Disodium 5'-guanylate	0627	—	1	—	—	
	Disodium 5'-inosinate	0631	—	1	—	—	
	Disodium 5'-ribonucleotides	0635	—	1	—	—	
	Ethyl maltol	0637	—	1	—	—	
	L-Glutamic acid	0620	—	1	—	—	
	5'-Guanylic acid	0626	—	1	—	—	
	5'-Inosinic acid	0630	—	1	—	—	
	Magnesium di-L-glutamate	0625	—	1	—	—	
	Monosodium L-glutamate	0624	—	1	—	—	
	Monopotassium L-glutamate	0622	—	1	—	—	
	Monosodium L-glutamate	0621	—	1	—	—	

Table 1 (continued)

Category	Additive name	INS No.	Limits (mg/kg)				
			Arsenic	Lead	Mercury	Cadmium	Other elements
Sweeteners	Alitame	0956	—	1	—	—	
	Aspartame	0951	—	1	—	—	
	Cyclohexylsulfamic acid	0952	—	1	—	—	Selenium <30
	Isomalt	0953	—	1	—	—	Nickel <2
	Lactitol	0966	—	1	—	—	Nickel <2
	Mannitol	0421	—	1	—	—	Nickel <2
	Saccharin and its sodium, potassium and calcium salts	0954	—	1	—	—	Selenium <30
	Sorbitol/sorbitol syrup	0420	—	1	—	—	Nickel <2
	Sucralose	0955	—	1	—	—	Nickel <2
	Xylitol	0967	—	1	—	—	
Thickeners	Ammonium alginate	0403	—	2	—	—	
	Ethyl cellulose	0462	—	2	—	—	
	Gum ghatti	0419	—	2	—	—	
	Hydroxypropyl cellulose	0463	—	2	—	—	
	Hydroxypropylmethyl cellulose	0464	—	2	—	—	
	Karaya gum	0416	—	2	—	—	
	Konjac flour	0425	—	2	—	—	
	Methylethyl cellulose	0465	—	2	—	—	
	Methyl cellulose	0461	—	2	—	—	
	Polyvinylpyrrolidone	1201	—	2	—	—	
	Powdered cellulose	0460(ii)	—	2	—	—	
	Tara gum	0417	—	2	—	—	
	Tragacanth gum	0413	—	2	—	—	

INS: International Numbering System.

Compendium of food additive specifications and its addenda (Annex 1, references 103, 109, 118, 124, 133, 139, 145 and 151).

In summary, the proposed changes to the current limits are as follows:

- The limits for arsenic are to be deleted, except in ferrocyanides of calcium, potassium and sodium, for which a limit of 3 mg/kg is proposed.
- The proposed limits for lead are 2 mg/kg in thickening agents and in the anticaking agent magnesium oxide, 1 mg/kg in flavour enhancers and sweeteners, 4 mg/kg in phosphates and 5 mg/kg in silicate and ferrocyanide anticaking agents.
- No limits were proposed for cadmium or mercury, as there was no concern that they are present in any of the substances under review.
- The limits for heavy metals (as lead) were deleted.

The Committee emphasized that the absence of a limit test for a particular metal from a specification which previously included the limit test for heavy metals (as lead) indicated that the level of contamination with that particular metal is so low as to be of no concern.

4. Flavouring agents

4.1 Substances evaluated by the Procedure for the Safety Evaluation of Flavouring Agents

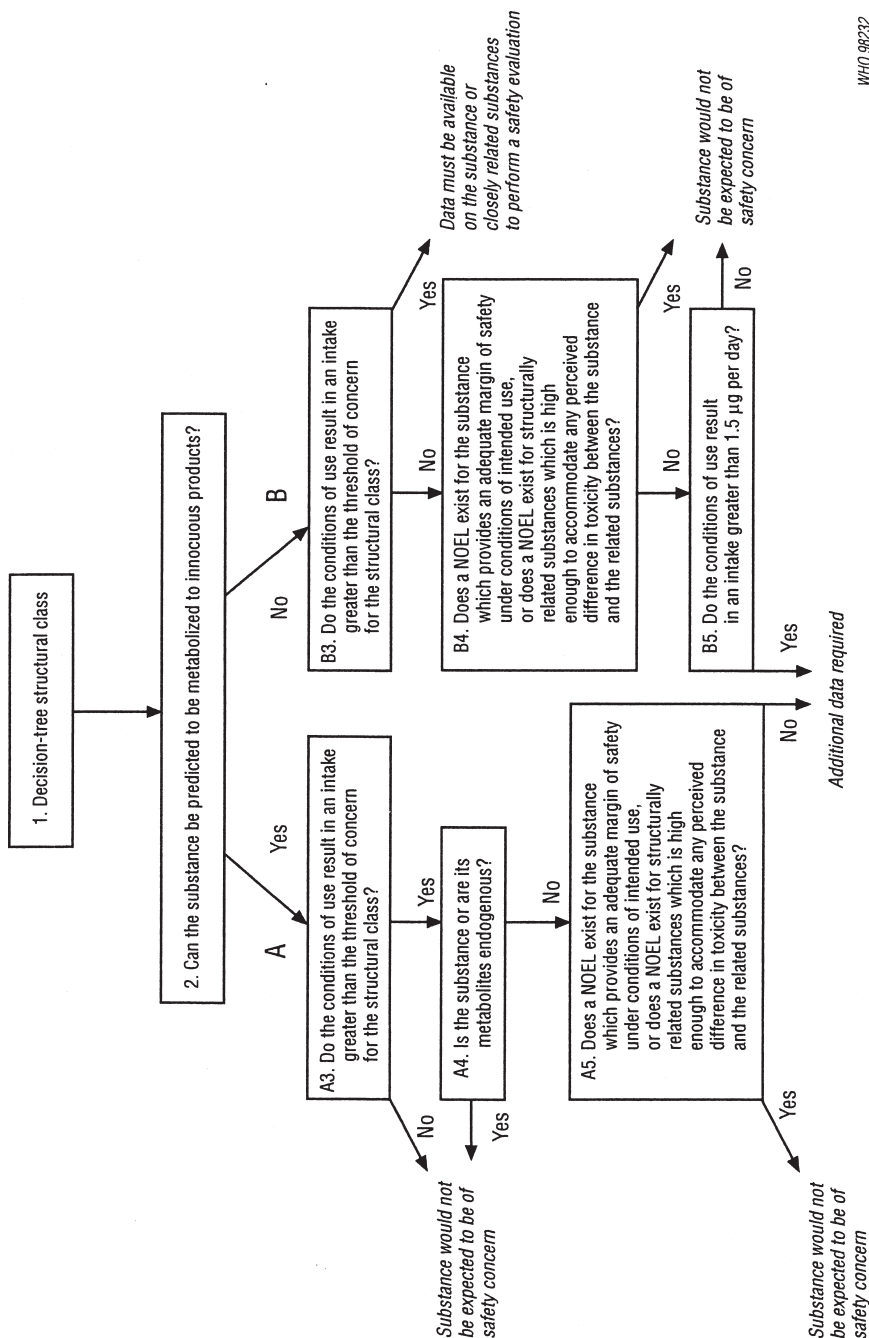
Six groups of flavouring agents were evaluated by the Procedure for the Safety Evaluation of Flavouring Agents, as outlined in Fig. 1 (Annex 1, references 116, 122, 131 and 137).

The Committee noted that, in applying the Procedure, a flavouring agent is first assigned to a structural class, as identified at the forty-sixth meeting (Annex 1, reference 122). The structural classes are as follows:

- Class I. Substances that have simple chemical structures and efficient modes of metabolism which would suggest a low order of toxicity when given by the oral route.
- Class II. Substances that have structural features that are less innocuous than those of substances in class I but are not suggestive of toxicity. Substances in this class may contain reactive functional groups.
- Class III. Substances that have structural features that permit no strong initial presumption of safety or may even suggest significant toxicity.

A key element of the Procedure involves determining whether a flavouring agent and the product(s) of its metabolism are innocuous and/or endogenous substances. For the purpose of the evaluations,

Procedure for the Safety Evaluation of Flavouring Agents



the Committee used the following definitions, adapted from the report of its forty-sixth meeting (Annex 1, reference 122):

Innocuous metabolic products are defined as products that are known or readily predicted to be harmless to humans at the estimated intake of the flavouring agent.

Endogenous substances are intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included. The estimated intake of a flavouring agent that is, or is metabolized to, an endogenous substance should be judged not to give rise to perturbations outside the physiological range.

Intake data

Estimates of the intake of flavouring agents by populations typically involve the acquisition of data on the amounts used in food. These data were derived from surveys in Europe and the USA. In Europe, a survey was conducted in 1995 by the International Organization of the Flavour Industry, in which flavour manufacturers reported the total amount of each flavouring agent that had been incorporated into food sold in the European Union during the previous year. Manufacturers were requested to exclude use of flavouring agents in pharmaceutical, tobacco or cosmetic products.

In the USA, a series of surveys was conducted between 1970 and 1987 by the National Research Council of the National Academy of Sciences (under contract to the Food and Drug Administration), in which information was obtained from ingredient manufacturers and food processors on the amount of each substance destined for addition to the food supply and on the usual and maximal levels at which each substance was added to foods in a number of broad categories.

In using the data from these surveys to estimate intakes of flavouring agents, the Committee assumed that only 60% of the total amount used in Europe and 80% of that used in the USA is reported and that the total amount used in food is consumed by only 10% of the population. Intake was thus calculated from the following equation:

$$\text{Intake} \left(\frac{\mu\text{g}}{\text{person per day}} \right) = \frac{\text{Annual volume of production (kg)} \times 10^9 (\mu\text{g/kg})}{\text{Population of consumers} \times 0.6 \text{ (or } 0.8) \times 365 \text{ days}}$$

The population of consumers was assumed to be 32×10^6 in Europe and 26×10^6 in the USA.

4.1.1 **Pyrazine derivatives**

The Committee evaluated a group of 41 flavouring agents consisting of pyrazine and pyrazine derivatives (see Table 2) by the Procedure

Table 2

Summary of results of the safety evaluations of pyrazine derivatives used as flavouring agents^a

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
Structural class II					
2-Methylpyrazine	761	109-08-0 	No Europe: 20 USA: 7	See note 1	No safety concern
2-Ethylpyrazine	762	13925-00-3 	No Europe: 3 USA: 6	See note 1	
2-Propylpyrazine	763	18138-03-9 	No Europe: 0.1 USA: 0.1	See note 1	
2-Isopropylpyrazine	764	29460-90-0 	No Europe: 0.1 USA: 0.1	See note 1	
2,3-Dimethylpyrazine	765	5910-89-4 	No Europe: 16 USA: 4	See note 1	
2,5-Dimethylpyrazine	766	123-32-0 	No Europe: 22 USA: 8	See note 1	
2,6-Dimethylpyrazine	767	108-50-9 	No Europe: 2 USA: 2	See note 1	

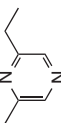
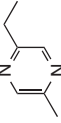
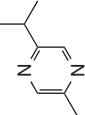
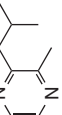
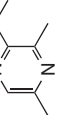
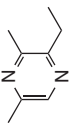
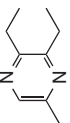
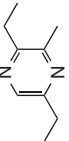
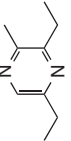
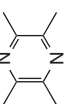
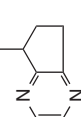
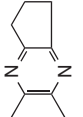
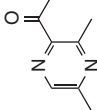
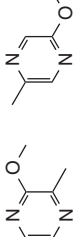
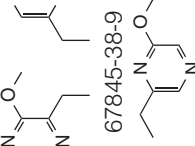
2-Ethyl-3-methylpyrazine	768	15707-23-0 	No Europe: 84 USA: 9	See note 1	}	No safety concern
2-Ethyl-6-methylpyrazine	769	13925-03-6 	No Europe: 0.4 USA: 0.4	See note 1		
2-Ethyl-5-methylpyrazine	770	13360-64-0 	No Europe: 5 USA: 1	See note 1		
2,3-Diethylpyrazine	771	15707-24-1 	No Europe: 2 USA: 1	See note 1		
2-Methyl-5-isopropylpyrazine	772	13925-05-8 	No Europe: ND USA: 0.4	See note 1		
2-Isobutyl-3-methylpyrazine	773	13925-06-9 	No Europe: 0.04 USA: 0.01	See note 1		
2,3,5-Trimethylpyrazine	774	14667-55-1 	No Europe: 120 USA: 46	See note 1		
2-Ethyl-3, (5 or 6)- dimethylpyrazine	775	13360-65-1 	No Europe: 44 USA: 9	See note 1		
		13925-07-0 				

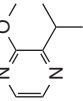
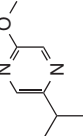
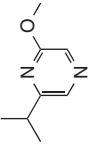
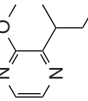
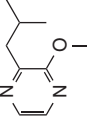
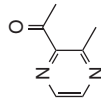
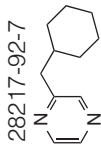
Table 2 (*continued*)

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
3-Ethyl-2,6-dimethylpyrazine	776	13925-07-0 	No Europe: 2 USA: 0.3	See note 1	No safety concern
2,3-Diethyl-5-methylpyrazine	777	18138-04-0 	No Europe: 0.2 USA: 1	See note 1	
2,5-Diethyl-3-methylpyrazine	778	32736-91-7 	No Europe: 0.01 USA: 0.01	See note 1	
3,5-Diethyl-2-methylpyrazine	779	18138-05-1 	No Europe: 0.01 USA: 0.01	See note 1	
2,3,5,6-Tetramethylpyrazine	780	1124-11-4 	No Europe: 8 USA: 19	See note 1	
5-Methyl-6,7-dihydro-5H-cyclopentapyrazine	781	23747-48-0 	No Europe: 5 USA: 4	See note 1	
6,7-Dihydro-2,3-dimethyl-5H-cyclopentapyrazine	782	38917-63-4 	No Europe: 0.01 USA: 0.01	See note 1	

Acetylpyrazine	784	22047-25-2		No Europe: 14 USA: 120	See note 2
2-Acetyl-3-ethylpyrazine	785	32974-92-8		No Europe: 1 USA: 0.1	See note 2
2-Acetyl-3,(5 or 6)-dimethylpyrazine	786	54300-08-2		No Europe: 1 USA: 1	See note 2
Methoxypyrazine	787	3149-28-8		No Europe: 4 USA: 1	See note 3
(2 or 5 or 6)-Methoxy-3-methylpyrazine	788	2847-30-5		No Europe: ND USA: 15	See note 3
2-Ethyl-(3 or 5 or 6)-methoxypyrazine	789	25680-58-4		No Europe: ND USA: 1	See note 3
		67845-38-9			

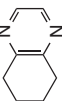
No safety concern

Table 2 (continued)

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
2-Methoxy-(3 or 5 or 6)-isopropylpyrazine	790	25773-40-4  56891-99-7  68039-46-3 	No Europe: ND USA: 0.1	See note 3	No safety concern
2-Methoxy-3-(1-methylpropyl)pyrazine	791	24168-70-5 	No Europe: 1 USA: 0.1	See note 3	
2-Isobutyl-3-methoxypyrazine	792	24683-00-9 	No Europe: 2 USA: 1	See note 3	
2-Acetyl-3-methylpyrazine	950	23787-80-6 	No Europe: 0.1 USA: 0.1	See note 2	
Structural class III (Cyclohexylmethyl)pyrazine	783	28217-92-7 	No Europe: ND USA: 0.01	See note 1	No safety concern

2-Methyl-(3 or 5 or 6)-ethoxypyrazine	793	<chem>CCOC1=CC=NC=C1C</chem> 32737-14-7 <chem>CCOC1=CC=NC=C1C</chem> 67845-34-5	No Europe: ND USA: 0.01	See note 3	No safety concern
		<chem>CCOC1=CC=NC=C1C</chem> 53163-97-6			
2-(Mercaptomethyl)pyrazine	794	<chem>SC1=CC=NC=C1</chem> 59021-02-2	No Europe: 0.01 USA: 0.01	See note 4	
2-Pyrazinylethane thiol	795	<chem>SCC1=CC=NC=C1</chem> 35250-53-4	No Europe: 0.2 USA: 1	See note 4	
Pyrazinylmethyl methyl sulfide	796	<chem>CSCC1=CC=NC=C1</chem> 21948-70-9	No Europe: ND USA: 0.01	See note 5	
(3 or 5 or 6)-(Methylthio)-2-methylpyrazine	797	<chem>CSC1=CC=NC=C1C</chem> 2882-20-4 <chem>CSC1=CC=NC=C1C</chem> 2884-14-2 <chem>CSC1=CC=NC=C1C</chem> 2884-13-1	No Europe: 7 USA: 13	See note 5	

Table 2 (*continued*)

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
5-Methylquinoxaline	798	13708-12-8 	No Europe: 26 USA: 1	See note 1	No safety concern
Pyrazine	951	290-37-9 	No Europe: 0.2 USA: 0.2	See note 1	
5,6,7,8-Tetrahydroquinoxaline	952	34413-35-9 	No Europe: 8 USA: ND	See note 1	

CAS: Chemical Abstracts Service; ND: no data on intake reported.

^a Step 2: All of the flavouring agents in this group are expected to be metabolized to innocuous products.

^b The thresholds for human intake for structural classes II and III are 540 µg/day and 90 µg/day, respectively. All intake values are expressed in µg/day.

Notes to Table 1

1. Detoxication by excretion in the urine unchanged, side-chain oxidation followed by conjugation and excretion, or ring hydroxylation followed by conjugation and excretion.
2. Detoxication as given in note 1 plus reduction to the corresponding alcohol and conjugation with glucuronic acid.
3. Detoxication as given in note 1 plus *O*-dealkylation followed by conjugation and excretion.
4. Detoxication as given in note 1 plus thiol oxidation, methylation, formation of mixed disulfides and conjugation with glucuronic acid.
5. Detoxication as given in note 1 plus *S*-oxidation to sulfoxide and sulfone analogues.

for the Safety Evaluation of Flavouring Agents (see Fig. 1). None of these agents has previously been evaluated by the Committee.

Thirty-four of the flavouring agents in this group are naturally occurring components of food. Members of this group have been detected in asparagus, potato, kohlrabi and wheaten bread.

4.1.1.1 Estimated daily per capita intake

The total annual volume of production of pyrazine and the 40 pyrazine derivatives in this group is approximately 2700kg in Europe and 2100kg in the USA. About 64% of the total annual volume of production in Europe is accounted for by 2,3,5-trimethylpyrazine (No. 774), 2-ethyl-3-methylpyrazine (No. 768) and 2-ethyl-3,(5 or 6)-dimethylpyrazine (No. 775). In the USA, about 66% of the total annual volume of production is accounted for by acetylpyrazine (No. 784), 2,3,5-trimethylpyrazine (No. 774) and 2,3,5,6-tetramethylpyrazine (No. 780). The estimated daily per capita intake of 2,3,5-trimethylpyrazine (No. 774) in Europe and of acetylpyrazine (No. 784) in the USA is about 120µg. The daily per capita intake of each agent in Europe and the USA is reported in Table 2.

4.1.1.2 Absorption, distribution, metabolism and elimination

Pyrazine is a weak base ($\log_{10}$ of the reciprocal of the dissociation constant, 13.4). Absorption of weak amine bases such as pyrazine derivatives is optimal at the pH of the intestine (5.0–7.0). In humans and laboratory rodents, orally administered substituted pyrazines are rapidly absorbed from the gut and excreted.

Alkyl-, alicyclic- and alkylaryl-substituted pyrazine derivatives. The biotransformation of alkyl-, alicyclic- and alkylaryl-substituted pyrazine derivatives (Nos 761–783 and 798) is expected to occur by oxidation of the alkyl side-chains. Methyl-substituted pyrazines are oxidized to yield the corresponding pyrazine-2-carboxylic acids. 5-Methylquinoxaline (No. 798) would be expected to be metabolized by the same pathway as the methyl- and ring-substituted pyrazine derivative 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), which is oxidized to yield the corresponding hydroxymethyl derivatives. An alternative pathway for the metabolism of pyrazine derivatives and the primary metabolic pathway for pyrazine (No. 951) itself involves hydroxylation of the pyrazine ring. Products of oxidative metabolism can be excreted unchanged or conjugated with glycine, glucuronic acid or sulfate before excretion.

Pyrazine derivatives containing an oxygenated functional group in the side-chain. In pyrazine derivatives containing a ring activator (e.g. a

methoxy substituent), significant ring hydroxylation may occur. Pyrazines with a methoxy side-chain, such as methoxypyrazine (No. 787), are more susceptible to nucleophilic attack, probably by molybdenum hydroxylases, and therefore primarily undergo ring hydroxylation. Additionally, the methoxy side-chain is *O*-demethylated. In rats, 3-acetylpyridine is reduced mainly to the secondary alcohol and excreted as the glucuronic acid conjugate. Therefore, acylated pyrazines (Nos 784–786 and 950) are expected to be metabolized mainly by reduction of the ketone functional group.

Pyrazine derivatives containing a thiol or sulfide functional group in the side-chain. Four pyrazine derivatives in this group contain either a thiol or a sulfide functional group in their side-chain. The possible metabolic pathways for the thiols, 2-(mercaptomethyl)pyrazine (No. 794) and 2-pyrazinylethane thiol (No. 795), include oxidation to form sulfinic acid (RSO_2H) and sulfonic acid (RSO_3H); methylation to yield methyl sulfides, which then form sulfoxides and sulfones; reaction with physiological thiols to form mixed disulfides and conjugation with glucuronic acid; or oxidation of the α -carbon, which results in desulfuration and formation of an aldehyde. Pyrazinylmethyl methyl sulfide (No. 796) and (3 or 5 or 6)-(methylthio)-2-methylpyrazine (No. 797) are predicted to be metabolized to sulfoxides and then to sulfones, which are the main urinary metabolites of simple sulfides. The Committee at its fifty-third meeting (Annex 1, reference 143) considered the pathways of metabolism of sulfur centres in its evaluation of a group of 137 flavouring agents that included aliphatic and aromatic sulfides and thiols, with and without an additional oxygenated functional group.

4.1.1.3 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents, the Committee assigned 32 of the 41 flavouring agents with one aromatic ring to structural class II on the basis of structural considerations and because they occur naturally (Nos 761–782, 784–792 and 950). Six flavouring agents with one aromatic ring were assigned to structural class III (Nos 783 and 793–797), as was 5,6,7,8-tetrahydroquinoxaline (No. 952). Pyrazine (No. 951) is the only agent in the group that bears no ring substituent, and it was therefore also assigned to structural class III. 5-Methylquinoxaline (No. 798) was assigned to structural class III because it is a polyheteroaromatic substance that does not contain sodium, potassium or calcium sulfonate or sulfamate.

Step 2. At current levels of intake, all 41 flavouring agents can be predicted to be metabolized to innocuous products, and the pathways

involved would not be expected to be saturated. The evaluation of these substances therefore proceeded via the left-hand side of the decision-tree.

Step A3. The estimated daily per capita intakes of all 32 flavouring agents in structural class II and all nine substances in structural class III are below the thresholds of concern for these classes (540 µg and 90 µg, respectively). The Committee concluded that these substances would not be expected to be of safety concern at the currently estimated levels of use.

Table 2 summarizes the evaluations of pyrazine and 40 pyrazine derivatives used as flavouring agents.

4.1.1.4 Consideration of combined intakes from use as flavouring agents

In the unlikely event that all 32 pyrazine derivatives in structural class II were to be consumed concurrently on a daily basis, the estimated combined intake would not exceed the threshold for human intake for this class (540 µg/day). In the unlikely event that all nine flavouring agents in structural class III were to be consumed concurrently on a daily basis, the estimated combined intake would not exceed the threshold for human intake for this class (90 µg/day). All the flavouring agents in this group are expected to be efficiently metabolized, and the available metabolic pathways would not be saturated. Evaluation of all the data indicated no safety concern associated with combined intake.

4.1.1.5 Conclusions

The Committee concluded that the safety of pyrazine and the 40 derivatives of pyrazine in this group would not raise concern at the currently estimated levels of intake.

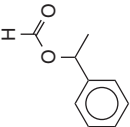
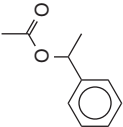
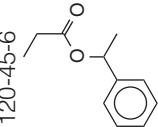
A monograph summarizing the data on the safety of this group of flavouring agents and specifications were prepared.

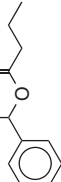
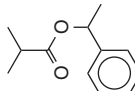
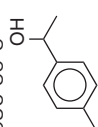
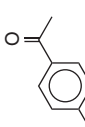
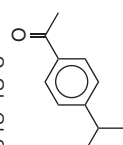
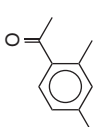
4.1.2 Aromatic substituted secondary alcohols, ketones and related esters

The Committee evaluated a group of flavouring agents that included α -methylbenzyl alcohol (No. 799), acetophenone (No. 806) and 36 structurally related aromatic secondary alcohols, ketones and related esters (Table 3) by the Procedure for the Safety Evaluation of Flavouring Agents (see Fig. 1). All the members of this group are considered to be aromatic secondary alcohols, ketones or related esters. The aromatic ring may contain additional alkyl substituents or a methoxy group, and the aliphatic side-chain may be unsaturated or

Table 3

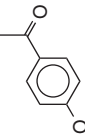
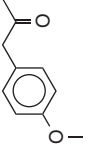
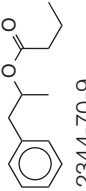
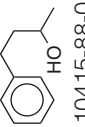
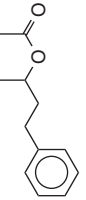
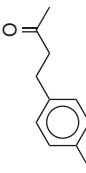
Summary of results of the safety evaluations of aromatic secondary alcohols, ketones and related esters used as flavouring agents^a

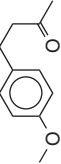
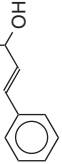
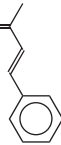
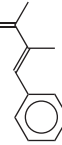
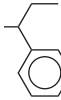
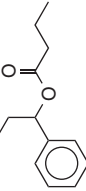
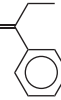
Flavouring agent	No.	CAS number and structure	Step A3/B3 ^b Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for substance or related substance?	Step B5 Does intake exceed 1.5µg/day?	Comments on predicted metabolism	Conclusions based on current intake
Structural class I							
α-Methylbenzyl alcohol ^c	799	98-85-1 	No Europe: 32 USA: 72	NR	NR	See note 1	No safety concern
α-Methylbenzyl formate	800	7775-38-4 	No Europe: 0.04 USA: 0.4	NR	NR	See note 2	
α-Methylbenzyl acetate	801	93-92-5 	No Europe: 200 USA: 650	NR	NR	See note 2	
α-Methylbenzyl propionate	802	120-45-6 	No Europe: 1 USA: 27	NR	NR	See note 2	

α -Methylbenzyl butyrate	803	3460-44-4 	No Europe: 1 USA: 0.01	NR	NR	See note 2
α -Methylbenzyl isobutyrate	804	7775-39-5 	No Europe: 29 USA: 1	NR	NR	See note 2
<i>p</i> , α -Dimethylbenzyl alcohol	805	536-50-5 	No Europe: 0.2 USA: 1	NR	NR	See note 1
Acetophenone	806	98-86-2 	No Europe: 18 USA: 170	NR	NR	See note 3
4-Methylacetophenone	807	122-00-9 	No Europe: 26 USA: 37	NR	NR	See note 3
<i>p</i> -Isopropylacetophenone	808	645-13-6 	No Europe: 0.01 USA: 0.4	NR	NR	See note 3
2,4-Dimethylacetophenone	809	89-74-7 	No Europe: 0.3 USA: 0.01	NR	NR	See note 3

No safety
concern

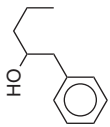
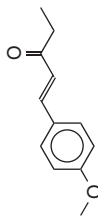
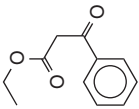
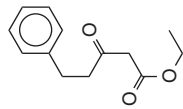
Table 3 (continued)

Flavouring agent	No.	CAS number and structure	Step A3/B3 ^b Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for substance or related substance?	Step B5 Does intake exceed 1.5µg/day?	Comments on predicted metabolism	Conclusions based on current intake
Acetanisole	810	100-06-1 	No Europe: 150 USA: 84	NR	NR	See notes 3 and 4	No safety concern
1-(<i>p</i> -Methoxyphenyl)-2-propanone	813	122-84-9 	No Europe: 0.2 USA: 0.1	NR	NR	See note 5	
α-Methylphenethyl butyrate	814	68922-11-2 	No Europe: 0.1 USA: 0.1	NR	NR	See note 6	
4-Phenyl-2-butanol	815	2344-70-9 	No Europe: 1 USA: 0.3	NR	NR	See note 7	
4-Phenyl-2-butyl acetate	816	10415-88-0 	No Europe: ND USA: 7	NR	NR	See note 7	
4-(<i>p</i> -Tolyl)-2-butanone	817	7774-79-0 	No Europe: 0.01 USA: 0.4	NR	NR	See notes 8 and 9	

4-(p-Methoxyphenyl)-2-butanone	818		No Europe: 5 USA: 840	NR	NR	See notes 8 and 9
4-Phenyl-3-buten-2-ol	819		No Europe: 2 USA: 0.1	NR	NR	See note 10
4-Phenyl-3-buten-2-one	820		No Europe: 3 USA: 7	NR	NR	See note 10
3-Methyl-4-phenyl-3-buten-2-one	821		No Europe: 0.1 USA: 0.1	NR	NR	See note 10
1-Phenyl-1-propanol	822		No Europe: 0.3 USA: 0.1	NR	NR	See note 11
α-Ethylbenzyl butyrate	823		No Europe: ND USA: 0.3	NR	NR	See note 2
Propiophenone	824		No Europe: 0.01 USA: 0.03	NR	NR	See note 11

No safety
concern

Table 3 (continued)

Flavouring agent	No.	CAS number and structure	Step A3/B3 ^b Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for substance or related substance?	Step B5 Does intake exceed 1.5µg/day?	Comments on predicted metabolism	Conclusions based on current intake
α-Propylphenethyl alcohol	825	705-73-7 	No Europe: 0.1 USA: 1	NR	NR	See note 6	No safety concern
1-(p-Methoxyphenyl)-1-penten-3-one	826	104-27-8 	No Europe: 0.5 USA: 110	NR	NR	See note 10	
Ethyl benzoylacetate	834	94-02-0 	No Europe: 0.01 USA: 140	NR	NR	See note 12	
Ethyl 2-acetyl-3-phenylpropionate	835	620-79-1 	No Europe: ND USA: 0.4	NR	NR	See note 12	

Structural class II

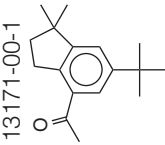
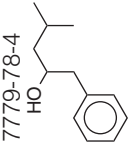
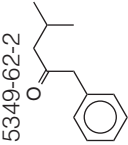
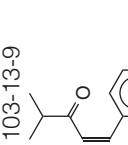
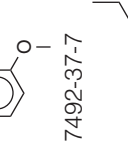
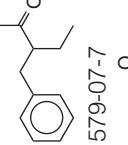
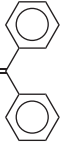
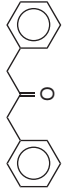
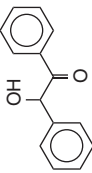
4-Acetyl-6- <i>tert</i> -butyl-1,1-dimethylindan	812		13171-00-1			No Europe: 6 USA: 1	No	Yes	Additional data required
α-Isobutylphenethyl alcohol	827		7779-78-4	No Europe: 29 USA: 3	NR	NR	See note 6	No safety concern	
4-Methyl-1-phenyl-2-pentanone	828		5349-62-2	No Europe: 10 USA: 0.3	NR	NR	See note 6		
1-(4-Methoxyphenyl)-4-methyl-1-penten-3-one	829		103-13-9	No Europe: 33 USA: 0.3	NR	NR	See note 10		
3-Benzyl-4-heptanone	830		7492-37-7	No Europe: ND USA: 1	NR	NR	See note 13		
1-Phenyl-1,2-propanedione	833		579-07-7	No Europe: 6 USA: 0.1	NR	NR	See note 14		

Table 3 (continued)

Flavouring agent	No.	CAS number and structure	Step A3/B3 ^b Does intake exceed the threshold for human intake?	Step B4 Adequate margin of safety for substance or related substance?	Step B5 Does intake exceed 1.5µg/day?	Comments on predicted metabolism	Conclusions based on current intake
Structural class III							
Methyl β-naphthyl ketone	811	93-08-3 	No Europe: 7 USA: 48	Yes The NOEL of 33 mg/kg of body weight per day in a 90-day study in rats is >10 000 times the estimated intake of methyl β-naphthyl ketone when used as a flavouring agent	NR	No safety concern	
Benzophenone	831	119-61-9 	No Europe: 27 USA: 11	NR	NR		
1,3-Diphenyl-2-propanone	832	102-04-5 	No Europe: 0.1 USA: 0.1	NR	NR		

Benzoin	836	119-53-9		No Europe: 7 USA: 21	NR	NR	See note 16	No safety concern

CAS: Chemical Abstracts Service; ND: no data on intake reported; NR: not required for evaluation.

^a Step 1: Twenty-eight of the flavouring agents in this group are in structural class I, six are in structural class II and four are in structural class III.

^b Step 2: All the flavouring agents in this group, except for Nos 811 and 812, are predicted to be metabolized to innocuous products.

^c The thresholds for human intake for structural classes I, II and III are 1800 µg/day, 540 µg/day and 90 µg/day, respectively. All intake values are expressed in µg/day.

^d An ADI of 0–0.1 mg/kg of body weight was established for α-methylbenzyl alcohol by the Committee at its forty-first meeting (Annex 1, reference 107), which was maintained at the present meeting.

Notes to Table 3

1. α-Methylbenzyl alcohol is conjugated with glucuronic acid and excreted in the urine. Oxidation of the methyl group to yield mandelic acid and hippuric acid may also occur.
2. α-Methylbenzyl esters are hydrolysed to simple aliphatic carboxylic acids and α-methylbenzyl alcohol. The acids are completely oxidized, and the alcohol is conjugated with glucuronic acid and excreted.
3. Acetophenone is reduced to α-methylbenzyl alcohol, conjugated with glucuronic acid and excreted primarily in the urine. The ketone may also undergo methyl group oxidation, eventually yielding mandelic acid and hippuric acid. These are also excreted in the urine.
4. Acetanilide may also undergo O-demethylation to yield the corresponding phenol. The phenol is excreted as the sulfate or glucuronic acid conjugate.
5. 1-(p-Methoxyphenyl)-2-propanone is reduced to the corresponding alcohol and excreted. O-Demethylation to yield the corresponding phenol may also occur.
6. α-Methylphenethyl butyrate is hydrolysed to butyric acid and α-methylphenethyl alcohol. The acid is completely metabolized and the alcohol is conjugated with glucuronic acid and excreted.
7. 4-Phenyl-2-butanol is conjugated with glucuronic acid and excreted. The corresponding acetate is predicted to be hydrolysed first to the alcohol and then to acetic acid.
8. 4-(p-Tolyl)-2-butanone is reduced to the corresponding alcohol. The alcohol may be conjugated with glucuronic acid and excreted primarily in the urine. The ketone may also undergo methyl group oxidation, eventually yielding a related carboxylic acid that is further oxidized to the phenylacetic acid derivative, which can be conjugated with glycine and excreted.
9. The alcohol may also be oxidized to the corresponding ketone and conjugated with glutathione.
10. 4-Phenyl-3-buten-2-one may be reduced to 4-phenyl-3-buten-2-ol. The alcohol is then further metabolized and excreted primarily as glycine conjugates. The ketone may also be conjugated with glutathione.
11. Propiophenone is reduced to the corresponding alcohol. The butyrate ester (No. 823) is predicted to be hydrolysed to the same alcohol (and butyric acid). The alcohol is conjugated with glucuronic acid and excreted primarily in the urine.
12. The esters are hydrolysed to ethanol and keto-carboxylic acids. The acids may be further oxidized and excreted as hippuric acid. The alcohol is completely metabolized.
13. Oxidation of side-chains is anticipated, primarily at the ω or ω-1 carbon atom. Carbonyl groups are reduced to alcohol, which can be conjugated with glucuronic acid. Oxidation of the side-chain can continue to benzoic or phenylacetic acid.
14. Reduced to the corresponding diol, which is conjugated with glucuronic acid.
15. Fifty per cent of a dose of 360 mg of benzophenone (No. 831) administered to rabbits by gavage was excreted as the glucuronide of the corresponding secondary alcohol within 48 h. 1,3-Diphenyl-2-propanone (No. 832), which is less sterically hindered than benzophenone, is also anticipated to be reduced to the alcohol and excreted as the glucuronide.
16. Benzoin is excreted as the glucuronic acid conjugate.

contain additional oxygenated functional groups. Of the 38 flavouring agents in this group, 34 are simple saturated or unsaturated methoxy- or alkyl-substituted benzene derivatives containing a secondary alcohol, corresponding ketone and/or related ester functional group.

The Committee had previously evaluated three members of this group. α -Methylbenzyl alcohol (No. 799) was evaluated at the forty-first meeting (Annex 1, reference 107), when an ADI of 0–0.1 mg/kg of body weight was established. At its twenty-fourth meeting, the Committee reviewed data on α -isobutylphenethyl alcohol (No. 827) (Annex 1, reference 53), and at its twenty-third and twenty-fifth meetings, it reviewed data on methyl β -naphthyl ketone (No. 811) (Annex 1, references 50 and 56). No ADI was allocated to either of these flavouring agents.

Of the 38 aromatic substituted secondary alcohols, ketones and related esters considered, 16 have been reported to occur naturally in foods. For instance, α -methylbenzyl alcohol (No. 799) has been detected in cheese, fruit and tea, and the corresponding ketone acetophenone (No. 806) is a natural component of berries, seafood, beef and nuts.

4.1.2.1 Estimated daily per capita intake

The total annual volume of production of the 38 aromatic secondary alcohols, ketones and related esters considered here is approximately 4.2 tonnes in Europe and 17 tonnes in the USA. Approximately 58% of the total annual volume of production in Europe is accounted for by α -methylbenzyl acetate (No. 801) and acetanisole (No. 810). The estimated daily per capita intakes of these two flavouring agents in Europe are 200 μ g and 150 μ g, respectively. In the USA, approximately 80% of the total volume of production arises from use of α -methylbenzyl acetate (No. 801), acetophenone (No. 806), 4-(*p*-methoxyphenyl)-2-butanone (No. 818) and ethyl benzoylacetate (No. 834). The estimated daily per capita intakes of these agents are 650 μ g, 170 μ g, 840 μ g and 140 μ g, respectively.

The estimated daily intake of each flavouring agent in the group is reported in Table 3.

4.1.2.2 Absorption, distribution, metabolism and elimination

Generally, the flavouring agents in this group are rapidly absorbed from the gut. The aromatic secondary alcohols (and aromatic ketones after reduction to the corresponding secondary alcohols) are then either conjugated with glucuronic acid and excreted primarily in the urine, or are further oxidized and excreted mainly as glycine conjugates. As aromatic esters are generally hydrolysed *in vivo* by the

catalytic activity of carboxylesterases, which are found predominantly in hepatocytes, it is anticipated that the 10 esters in this group of flavouring agents will be hydrolysed to their parent aromatic or aliphatic alcohols and carboxylic acids. The eight aromatic secondary alcohols formed as a result of this process are excreted as their glucuronides or are further metabolized and excreted in the urine. The corresponding eight simple aliphatic carboxylic acids are metabolized completely by well-known pathways. The two remaining esters (Nos 834 and 835) are hydrolysed to ethanol and aromatic keto-carboxylic acids (3-oxo-3-phenylpropanoic acid and 3-oxo-5-phenylpentanoic acid, respectively), which are anticipated to be further metabolized and excreted in the urine, like other aromatic ketones.

Simple aromatic ring substitution with methyl, isopropyl or methoxy groups (Nos 805, 807–810, 813, 817, 818, 826 and 829) is predicted to have little effect on the principal metabolic pathways. It is more difficult to predict the metabolic fate of Nos 811 and 812 on the basis of the available data, as it is not known to what extent they are distributed in the tissues and eliminated. One of these substances, 4-acetal-6-*tert*-butyl-1,1-dimethylindan (No. 812), might accumulate in human adipose tissue.

4.1.2.3 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents (see Fig. 1) to these 38 aromatic flavouring agents, the Committee assigned 28 to structural class I (Nos 799–810, 813–826, 834 and 835). Six flavouring agents were assigned to structural class II, one (No. 833) because it is a vicinal diketone and the other five because they contain a fused non-aromatic carbocyclic ring (No. 812) or aliphatic substituent chains with more than five carbon atoms (Nos 827–830). Four of the agents (Nos 811, 831, 832 and 836) were assigned to structural class III because they contain more than one aromatic ring and cannot be hydrolysed to mononuclear residues.

Step 2. At current levels of estimated intake, 36 of the 38 flavouring agents in this group are predicted to be metabolized to innocuous products and the available metabolic pathways would not be expected to be saturated. Evaluation of these substances therefore proceeded via the left-hand side of the decision-tree. The two remaining flavouring agents (Nos 811 and 812) cannot be predicted to be metabolized to innocuous products, and therefore their evaluation proceeded via the right-hand side of the decision-tree.

Step A3. The estimated daily per capita intakes of the 28 flavouring agents in structural class I, five of the six flavouring agents in

structural class II and three of the four agents in structural class III are below the thresholds of concern for these classes (1800 µg, 540 µg and 90 µg, respectively). The Committee concluded that these substances would not be expected to be of safety concern at their currently estimated levels of use as flavouring agents.

Step B3. The estimated daily per capita intakes of one agent in structural class II (No. 812) and one in structural class III (No. 811) are below the thresholds of concern for these classes (540 µg and 90 µg, respectively).

Step B4. The NOEL identified for methyl β-naphthyl ketone (No. 811) in a 90-day study in rats treated orally was the highest dose tested, 33 mg/kg of body weight per day. This dose provided safety margins >100 000 and >10 000 times the estimated daily per capita intakes in Europe and in the USA, respectively. The Committee concluded that methyl β-naphthyl ketone does not pose a safety concern at currently estimated levels of use as a flavouring agent.

No data were available on the toxicity of the remaining agent (No. 812) or of relevant structurally related substances. Accordingly, the evaluation of this substance proceeded to step B5.

Step B5. As the estimated daily per capita intake of 4-acetyl-6-*tert*-butyl-1,1-dimethylindan (No. 812) in Europe (6 µg) exceeds the threshold of 1.5 µg/person per day, further data are required for a safety evaluation. The Committee concluded that this flavouring agent cannot be classified as of “no safety concern at current level of intake”.

Table 3 summarizes the evaluations of α-methylbenzyl alcohol and acetophenone and 36 structurally related flavouring agents.

4.1.2.4 Consideration of combined intakes from use as flavouring agents

In the unlikely event that all foods containing all the flavouring agents in structural classes I and II were to be consumed simultaneously on a daily basis, the estimated combined intake would exceed the human intake threshold for class II (540 µg). However, the agents are expected to be metabolized efficiently and the available metabolic pathways would not be saturated. Evaluation of all the data indicated no safety concern associated with combined intake.

4.1.2.5 Conclusions

The Committee concluded that 37 of this group of 38 aromatic secondary alcohols, ketones and related esters would not pose a safety concern at currently estimated levels of use as flavouring agents.

The Committee noted that when data on toxicity were available, they were consistent with the results of the safety evaluation. Data on toxicity were required for two agents (Nos 811 and 812) in application of the Procedure. Relevant data were available for one of these substances (No. 811), which gave a large safety margin in relation to the estimated intake.

The Committee required additional data to evaluate the safety of 4-acetyl-6-*tert*-butyl-1,1-dimethylindan (No. 812), which could not be predicted to be metabolized to innocuous products, for which satisfactory data on toxicity were not available and of which the estimated daily intake, 6µg/person in Europe, exceeded the threshold of 1.5µg/person per day.

A monograph summarizing the safety data on this group of flavouring agents was prepared.

4.1.3 ***Benzyl derivatives***

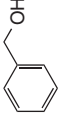
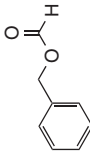
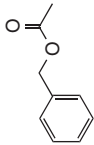
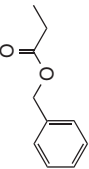
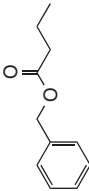
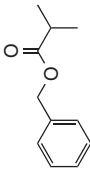
The Committee evaluated a group of 37 flavouring agents¹ that consisted of benzyl alcohol (No. 25), benzaldehyde (No. 22), benzoic acid (No. 850) and related substances (Table 4) by the Procedure for the Safety Evaluation of Flavouring Agents (see Fig. 1). All members of this group are aromatic primary alcohols, aldehydes, carboxylic acids or related esters or acetals. The benzene ring may be ring-substituted with alkyl substituents (Nos 863–869).

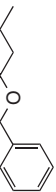
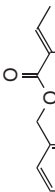
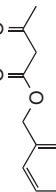
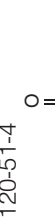
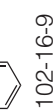
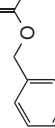
The Committee had previously evaluated five members of the group. Benzyl alcohol (No. 25) was evaluated at the twenty-third and forty-sixth meetings (Annex 1, references 50 and 122); benzyl acetate (No. 23) was evaluated at the eleventh, twenty-seventh, twenty-ninth, thirty-first, thirty-fifth, forty-first and forty-sixth meetings (Annex 1, references 14, 62, 70, 77, 88, 107 and 122); benzyl benzoate (No. 24) was evaluated at the fifteenth and twenty-third meetings (Annex 1, references 26 and 50); benzaldehyde (No. 22) was evaluated at the eleventh and forty-sixth meetings (Annex 1, references 14 and 122; and benzoic acid (No. 850) was evaluated at the sixth, ninth, seventeenth, twenty-seventh and forty-sixth meetings (Annex 1, references 6, 11, 32, 62 and 122). At its forty-sixth meeting, the Committee evaluated benzyl acetate, benzyl alcohol, benzaldehyde, benzoic acid and the benzoate salts (calcium, potassium and sodium) as a group and maintained the group ADI of 0–5mg/kg of body weight as benzoic acid equivalents (Annex 1, reference 122).

¹ During evaluation of these flavouring agents, the Committee questioned whether some substances in this group (Nos 850, 861 and 862) were used as flavouring agents and therefore appropriately evaluated by the Procedure. Information to address this question will be sought from the relevant manufacturers.

Table 4

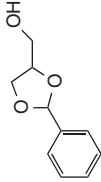
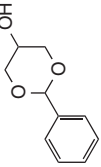
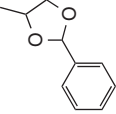
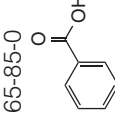
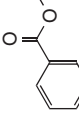
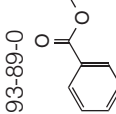
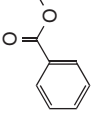
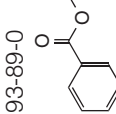
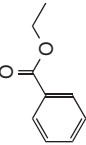
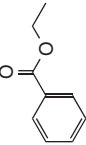
Summary of results of the safety evaluations of benzyl derivatives^a

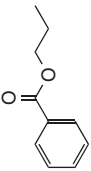
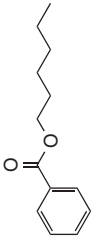
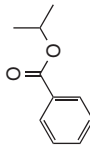
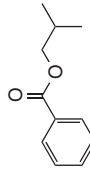
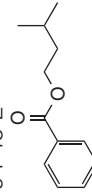
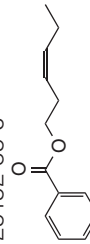
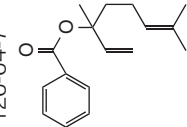
Flavouring agent	No.	CAS number and structure	Step A3/B3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Step B4 Adequate margin of safety for substance or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Benzyl alcohol ^c	25	100-51-6 	Yes Europe: 16000 USA: 17000	Yes		See note 1	
Benzyl formate	841	104-57-4 	No Europe: 41 USA: 51	NR		See note 2	
Benzyl acetate ^c	23	140-11-4 	No Europe: 1400 USA: 850	NR		See note 2	
Benzyl propionate	842	122-63-4 	No Europe: 49 USA: 99	NR		See note 2	No safety concern
Benzyl butyrate	843	103-37-7 	No Europe: 120 USA: 290	NR		See note 2	
Benzyl isobutyrate	844	103-28-6 	No Europe: 15 USA: 21	NR		See note 2	

Benzyl isovalerate	845	103-38-8		No Europe: 14 USA: 19	NR	See note 2
Benzyl <i>trans</i> -2-methyl-2-butenate	846	37526-88-8		No Europe: 0.01 USA: 0.03	NR	See note 2
Benzyl 2,3-dimethylcrotonate	847	7492-69-5		No Europe: 0.01 USA: 1	NR	See note 2
Benzyl acetoacetate	848	5396-89-4		No Europe: 0.2 USA: 0.07	NR	See note 2
Benzyl benzoate ^c	24	120-51-4		Yes Europe: 1900 USA: 4200	Yes	See notes 2 and 6
Benzyl phenylacetate	849	102-16-9		No Europe: 5 USA: 57	NR	See note 2
Benzaldehyde ^c	22	100-52-7		Yes Europe: 9300 USA: 36000	Yes	See note 3
Benzaldehyde dimethyl acetal	837	1125-88-8		No Europe: 0.2 USA: 0.3	NR	See note 4

No safety concern

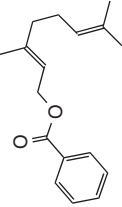
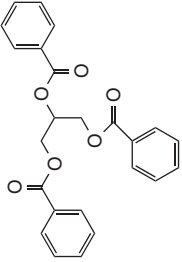
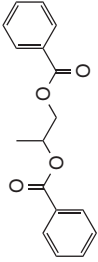
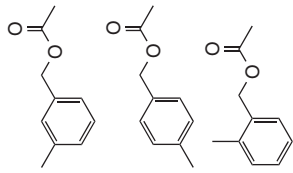
Table 4 (continued)

Flavouring agent	No.	CAS number and structure	Step A3/B3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Step B4 Adequate margin of safety for substance or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Benzaldehyde glyceryl acetal	838	1319-88-6 	No Europe: 16 USA: 300	NR	See note 4		No safety concern
							
Benzaldehyde propylene glycol acetate	839	2568-25-4 	No Europe 0.04 USA: 110	NR	See note 4		Evaluation not finalized
							
Benzoic acid ^{c,d}	850	65-85-0 	No Europe: 39 USA: 340	NR	See note 5		No safety concern
							
Methyl benzoate	851	93-58-3 	No Europe: 47 USA: 230	NR	See note 6		No safety concern
							
Ethyl benzoate	852	93-89-0 	No Europe: 110 USA: 110	NR	See note 6		No safety concern
							

Propyl benzoate	853	2315-68-6		No Europe: 0.01 USA: 0.3	NR	See note 6
Hexyl benzoate	854	6789-88-4		No Europe: 380 USA: 1	NR	See note 6
Isopropyl benzoate	855	939-48-0		No Europe: 0.004 USA: 0.3	NR	See note 6
Isobutyl benzoate	856	120-50-3		No Europe: 0.4 USA: 1	NR	See note 6
Isoamyl benzoate	857	94-46-2		No Europe: 110 USA: 33	NR	See note 6
<i>cis</i> -3-Hexenyl benzoate	858	25152-85-6		No Europe: 8 USA: 0.1	NR	See note 6
Linalyl benzoate	859	126-64-7		No Europe: 10 USA: 2	NR	See note 6

No safety
concern

Table 4 (continued)

Flavouring agent	No.	CAS number and structure	Step A3/B3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Step B4 Adequate margin of safety for substance or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Geranyl benzoate	860	94-48-4 	No Europe: 4 USA: 0.03	NR		See note 6	No safety concern
Glyceryl tribenzoate ^d	861	614-33-5 	No Europe: ND USA: 49	NR		See note 6	Evaluation not finalized
Propylene glycol dibenzoate ^d	862	19224-26-1 	No Europe: ND USA: 14	NR		See note 6	
Methylbenzyl acetate (mixed <i>ortho</i> -, <i>meta</i> - and <i>para</i> -isomers)	863	29759-11-3 	No Europe: ND USA: 3	NR		See note 2	No safety concern

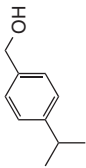
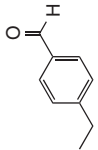
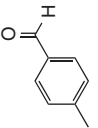
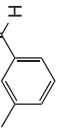
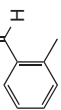
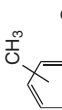
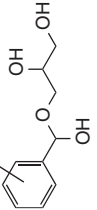
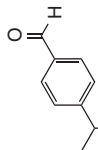
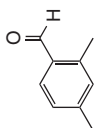
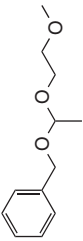
<i>p</i>-Isopropylbenzyl alcohol	864	536-60-7		No Europe: 0.3 USA: 0.3	NR	See note 1
	865	4748-78-1		No Europe: 0.4 USA: 6	NR	See note 3
	866	1334-78-7		No Europe: 260 USA: 1100	NR	See note 3
Tolualdehydes (mixed <i>ortho</i>-, <i>meta</i>- and <i>para</i>-isomers)				No safety concern		
						
						
Tolualdehyde glyceryl acetal	867	1333-09-1		No Europe: 0.01 USA: 1	NR	See note 4
	868	122-03-2		No Europe: 130 USA: 1	NR	See note 3
2,4-Dimethylbenzaldehyde	869	15764-16-6		No Europe: 0.4 USA: 0.1	NR	See note 3

Table 4 (continued)

Flavouring agent	No.	CAS number and structure	Step A3/B3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Step B4 Adequate margin of safety for substance or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Benzyl 2-methoxyethyl acetal	840	7492-39-9 	No Europe: ND USA: 1		Yes The NOEL of 6 mg/kg of body weight per day in a two-generation study of reproductive toxicity in rats is > 10000 times the estimated intake of benzyl 2-methoxyethyl acetal when used as a flavouring agent	See note 7	No safety concern

CAS: Chemical Abstracts Service; ND: no data on intake reported; NR: not required for evaluation because consumption of the substance was determined to be of no safety concern at step A3 of the Procedure.

- ^a Step 1: All of the flavouring agents in this group are in structural class I.
^b Step 2: All of the flavouring agents in this group are expected to be metabolized to innocuous products, except for benzyl 2-methoxyethyl acetal (No. 840). The threshold for human intake for structural class I is 1800 µg/day. All intake values are expressed in µg/day.
^c A group ADI of 0–5 mg/kg of body weight for benzoic acid, the benzoate salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate and benzyl alcohol, expressed as benzoic acid equivalents, was confirmed by the Committee at its forty-sixth meeting (Annex 1, reference 122) and extended to include benzyl benzoate at the present meeting.
^d Further information is required to determine whether this substance is in current use as a flavouring agent.

Notes to Table 4

1. Benzyl alcohols are oxidized to the corresponding acids, which are conjugated with glycine and excreted as hippuric acid.
2. Benzyl esters are hydrolysed to the corresponding acids and alcohols.
3. Benzyl aldehydes are oxidized to the corresponding acids.
4. Benzaldehyde acetals are hydrolysed to yield the aldehyde.
5. Benzoic acid is conjugated with glycine and excreted as hippuric acid.
6. Benzoate esters are hydrolysed to yield the corresponding alcohols and acids.
7. Hydrolysed to acetaldehyde, benzyl alcohol and 2-methoxyethanol.

Of the 37 substances in this group, 29 have been reported to occur naturally in foods. They have been detected in a wide variety of fruits, vegetables, meats, cheeses and wine.

4.1.3.1 Estimated daily per capita intake

The total annual volume of production of the 37 benzyl derivatives in this group is approximately 210 tonnes in Europe and 460 tonnes in the USA. About 91% of the total annual volume of production in Europe and 94% of that in the USA is accounted for by benzyl alcohol (No. 25), benzaldehyde (No. 22) and benzyl benzoate (No. 24). About 31% of the total annual volume of production in Europe is accounted for by benzaldehyde, 54% by benzyl alcohol and 6% by benzyl benzoate. About 59% of the total annual volume of production in the USA is accounted for by benzaldehyde, 28% by benzyl alcohol and 7% by benzyl benzoate. The estimated daily intake per capita of these three agents in Europe is 9300µg of benzaldehyde, 16000µg of benzyl alcohol and 1900µg of benzyl benzoate. The estimated daily intake per capita in the USA is 36000µg of benzaldehyde, 17000µg of benzyl alcohol and 4200µg of benzyl benzoate. The estimated daily per capita intake of each flavouring agent in Europe and the USA is reported in Table 4.

Benzoic acid is not only present in food and flavours but is also endogenous in the human body. Endogenous benzoic acid is formed through the phenylalanine–tyrosine pathway (Annex 1, reference 123).

4.1.3.2 Absorption, distribution, metabolism and elimination

In general, aromatic esters are hydrolysed *in vivo* by the catalytic activity of carboxylesterases, which are found predominantly in hepatocytes. All the benzyl and benzoate esters and acetals of benzaldehyde (or acetaldehyde) are anticipated to be hydrolysed readily under acidic conditions to yield benzyl alcohol (and carboxylic acids) and to benzaldehyde (and alcohols), respectively, followed by oxidation to yield benzoic acid. Benzoate esters are hydrolysed to benzoic acid (and alcohols). The remaining alcohol or acid components formed by hydrolysis are simple aliphatic substances, which are either oxidized to polar metabolites and excreted or metabolized in the fatty acid pathway and tricarboxylic acid cycle.

Benzyl derivatives have been shown to be absorbed rapidly in the gut, metabolized primarily in the liver and excreted in the urine as glycine conjugates of benzoic acid derivatives. Once absorbed, benzyl derivatives are oxidized and excreted primarily as the glycine conjugate of benzoic acid (hippurate). When high doses of benzyl derivatives are

given, formation of the glycine conjugate is limited; when glycine is depleted, free benzoic acid may sequester acetyl coenzyme A or be excreted unchanged or as the glucuronic acid conjugate. Aromatic ring substitution is anticipated to have little effect on the principal pathway of metabolism.

Oxidation of the alcohol or aldehyde group may be accompanied by oxidation of the alkyl side-chain.

4.1.3.3 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents (see Fig. 1) to the 37 benzyl derivatives, the Committee assigned all of them to structural class I.

Step 2. At currently estimated levels of intake, 36 of the 37 substances in this group can be predicted to be metabolized to innocuous products. The evaluation of these substances therefore proceeded via the left-hand side of the decision-tree. One compound, benzyl 2-methoxyethyl acetal (No. 840), cannot be predicted to be metabolized to innocuous products, and its evaluation therefore proceeded via the right-hand side of the decision-tree.

Step A3. The estimated daily per capita intakes of 33 of the flavouring agents in this group are below the threshold of concern for structural class I (1800µg). The Committee concluded that these substances would not be expected to be of safety concern at current estimated levels of use as flavouring agents. The estimated daily per capita intakes of the remaining three substances are above the threshold of concern for this class, that of benzyl alcohol (No. 25) being 16 000µg in Europe and 17 000µg in the USA, that of benzyl benzoate (No. 24) being 1900µg in Europe and 4200µg in the USA, and that of benzaldehyde (No. 22) being 9300µg in Europe and 36 000µg in the USA. Accordingly, the evaluation of these three substances proceeded to step A4.

Step A4. Benzyl alcohol, benzyl benzoate and benzaldehyde are readily metabolized to benzoic acid, which is endogenous in humans. These agents would therefore not be expected to be of safety concern.

Step B3. For benzyl 2-methoxyethyl acetal (No. 840), no data on intake were reported for Europe and an intake of 1µg/person per day was reported for the USA, which is below the threshold of concern for substances in structural class I (1800µg/person per day). The evaluation of this substance therefore proceeded to step B4.

Step B4. The NOEL of 6 mg/kg of body weight per day for benzyl 2-methoxyethyl acetal (No. 840) in a two-generation study of reproductive toxicity in rats provides a margin of safety >10000 times the estimated daily per capita intake in the USA. The Committee concluded that this substance would not pose a safety concern at the currently estimated level of intake.

Table 4 summarizes the evaluations of the 37 benzyl derivatives used as flavouring agents.

4.1.3.4 Consideration of combined intakes from use as flavouring agents

In the unlikely event that all the benzyl derivatives used as flavouring agents, except benzyl 2-methoxyethyl acetal (No. 840), were to be consumed concurrently on a daily basis, the estimated combined intake would exceed the threshold for human intake for structural class I. However, these agents are expected to be efficiently detoxicated and the available detoxication pathways would not be saturated. Evaluation of all the data indicated no safety concern associated with combined intake.

Furthermore, the total combined daily intake per kilogram of body weight of all benzyl derivatives (0.5 mg in Europe and 1 mg in the USA) is lower than the group ADI of 0–5 mg/kg of body weight for benzoic acid, the benzoate salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate and benzyl alcohol, expressed as benzoic acid equivalents, which was maintained by the Committee at its forty-sixth meeting (Annex 1, reference 122). The three benzyl derivatives that account for more than 90% of the total intake of this group of substances in Europe and the USA are benzyl benzoate (No. 24), which is rapidly hydrolysed to benzyl alcohol and benzoic acid, benzaldehyde (No. 22) and benzyl alcohol (No. 25). All these substances are readily metabolized to benzoic acid, which is endogenous in humans. The Committee considered that the endogenous concentration of this substance would not give rise to perturbations outside the physiological range. Therefore, these three substances were considered to be of no safety concern at currently estimated levels of intake.

4.1.3.5 Conclusions

The Committee concluded that the safety of the flavouring agents in the group of benzyl derivatives would not present concern at currently estimated levels of use as flavouring agents. No data on toxicity were required in application of the Procedure to 36 of the 37 benzyl derivatives in the group, and the Committee noted that the available information was consistent with the results of the safety evaluation.

The necessary data on toxicity were available for benzyl 2-methoxyethyl acetal (No. 840).

A monograph summarizing the safety data on this group of flavouring agents and specifications were prepared.

4.1.4 ***Hydroxy- and alkoxy-substituted benzyl derivatives***

The Committee evaluated a group of flavouring agents¹ comprising 46 structurally related substances by the Procedure for the Safety Evaluation of Flavouring Agents (see Fig. 1). All members of this group are aromatic primary alcohols, aldehydes, carboxylic acids or their corresponding esters or acetals. The structural feature common to all members of the group is a primary oxygenated functional group bound directly to a benzene ring. The ring also contains hydroxy or alkoxy substituents (see Table 5).

The Committee had previously evaluated four members of this group. Ethyl vanillin (No. 893) was evaluated by the Committee at its eleventh meeting (Annex 1, reference 14), when a conditional ADI of 0–10 mg/kg of body weight was established. At its thirty-fifth meeting, the Committee converted this ADI to a temporary ADI of 0–5 mg/kg of body weight (Annex 1, reference 88). At its thirty-ninth meeting, the Committee extended the temporary ADI (Annex 1, reference 101). At its forty-fourth meeting, the Committee allocated an ADI of 0–3 mg/kg of body weight to ethyl vanillin (Annex 1, reference 116). Vanillin (No. 889) was evaluated by the Committee at its eleventh meeting (Annex 1, reference 14), when an ADI of 0–10 mg/kg of body weight was established. Methyl salicylate (No. 899) was evaluated by the Committee at its eleventh meeting (Annex 1, reference 14), and an ADI of 0–0.5 mg/kg of body weight was established. Piperonal (No. 896) was also evaluated at the eleventh meeting, and an ADI of 0–2.5 mg/kg of body weight was established (Annex 1, reference 14).

Twenty-nine of the 46 substances in this group of flavouring agents have been reported to occur naturally in food. Vanillin (No. 889), a major constituent of natural vanilla, is also present in strawberries and milk. Methyl salicylate (No. 899), the predominant substituent of oil of wintergreen, is also found in tomatoes and grilled beef. Ethyl vanillin (No. 893) has been detected in raspberries and ginger, while piperonal (No. 896) is found in cooked chicken and pepper.

¹ During evaluation of these flavouring agents, the Committee questioned whether one substance in this group (No. 870) was currently used as a flavouring agent and therefore appropriately evaluated by the Procedure. Information to address this question will be sought from relevant manufacturers.

Table 5

Summary of results of the safety evaluations of hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents^a

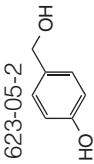
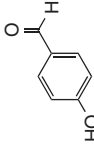
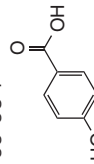
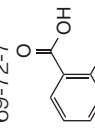
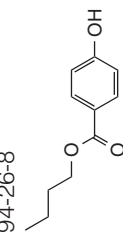
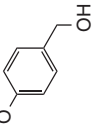
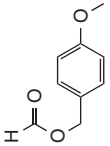
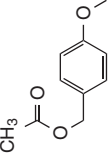
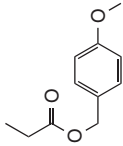
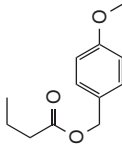
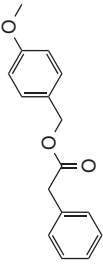
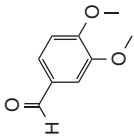
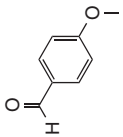
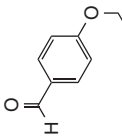
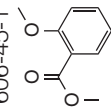
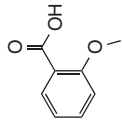
Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Step A5 Adequate margin of safety for substance or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class I							
4-Hydroxybenzyl alcohol	955		No Europe: 6 USA: 0.06	NR	NR	See note 1	No safety concern
4-Hydroxybenzaldehyde	956		No Europe: 64 USA: 59	NR	NR	See note 1	
4-Hydroxybenzoic acid	957		No Europe: 19 USA: 17	NR	NR	See note 1	
2-Hydroxybenzoic acid	958		No Europe: 0.03 USA: 0.03	NR	NR	See note 1	
Butyl <i>p</i> -hydroxybenzoate ^c	870		No Europe: ND USA: 0.03	NR	NR	See note 2	Evaluation not finalized

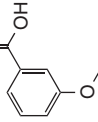
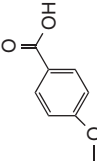
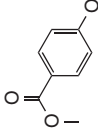
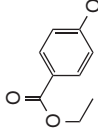
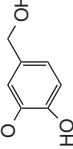
Table 5 (continued)

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Step A5 Adequate margin of safety for substance or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Anisyl alcohol	871	105-13-5 	No Europe: 150 USA: 58	NR	NR	See note 1	No safety concern
Anisyl formate	872	122-91-8 	No Europe: 46 USA: 24	NR	NR	See note 2	
Anisyl acetate	873	104-21-2 	No Europe: 59 USA: 300	NR	NR	See note 2	
Anisyl propionate	874	7549-33-9 	No Europe: ND USA: 5	NR	NR	See note 2	
Anisyl butyrate	875	6963-56-0 	No Europe: 34 USA: 0.1	NR	NR	See note 2	

Anisyl phenylacetate	876	102-17-0		No Europe: 0.003 USA: 0.1	NR	NR	See note 3
Veratraldehyde	877	120-14-9		No Europe: 140 USA: 55	NR	NR	See note 1
p-Methoxybenzaldehyde	878	123-11-5		No Europe: 440 USA: 580	NR	NR	See note 1
p-Ethoxybenzaldehyde	879	10031-82-0		No Europe: 0.1 USA: 0.01	NR	NR	See note 1
Methyl o-methoxybenzoate	880	606-45-1		No Europe: 57 USA: 8	NR	NR	See note 2
2-Methoxybenzoic acid	881	579-75-9		No Europe: ND USA: 0.01	NR	NR	See note 1

No safety concern

Table 5 (continued)

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Step A5 Adequate margin of safety for substance or related substance?	Comments on predicted metabolism	Conclusion based on current intake
3-Methoxybenzoic acid	882	586-38-9 	No Europe: ND USA: 0.01	NR	NR	See note 1	No safety concern
4-Methoxybenzoic acid	883	100-09-4 	No Europe: ND USA: 0.1	NR	NR	See note 1	
Methyl anisate	884	121-98-2 	No Europe: 1 USA: 0.01	NR	NR	See note 2	
Ethyl p-anisate	885	94-30-4 	No Europe: 11 USA: 2	NR	NR	See note 2	
Vanillyl alcohol	886	498-00-0 	No Europe: 6 USA: 6	NR	NR	See note 1	

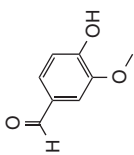
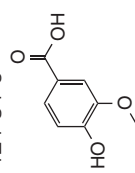
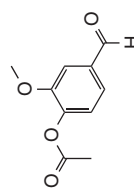
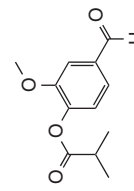
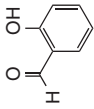
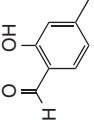
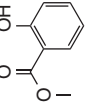
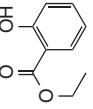
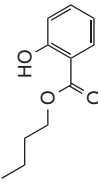
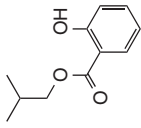
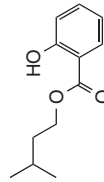
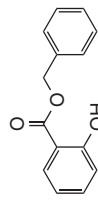
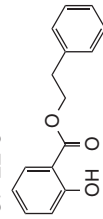
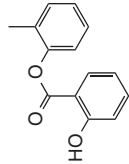
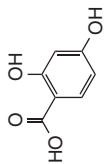
Vanillin ^d	889	121-33-5		Yes Europe: 55 000 USA: 150 000	No	Yes The NOEL of 1000mg/kg of body weight per day in a 2-year study in rats is >100 times the estimated daily intake of vanillin when used as a flavouring agent NR	See note 1	No safety concern
4-Hydroxy-3-methoxybenzoic acid	959	121-34-6		No Europe: 29 USA: 26	NR	NR	See note 1	
Vanillin acetate	890	881-68-5		No Europe: 2 USA: 1	NR	NR	See note 2	
Vanillin isobutyrate	891	20665-85-4		No Europe: 64 USA: 0.04	NR	NR	See note 2	

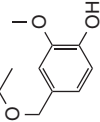
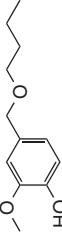
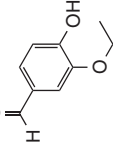
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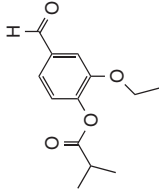
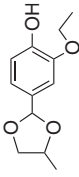
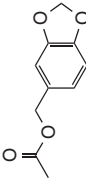
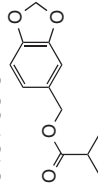
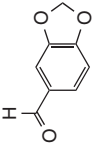
Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Step A5 Adequate margin of safety for substance or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Salicylaldehyde	897	90-02-8 	No Europe: 98 USA: 16	NR	NR	See note 1	No safety concern
2-Hydroxy-4-methylbenzaldehyde	898	698-27-1 	No Europe: 1 USA: 0.3	NR	NR	See note 1	
Methyl salicylate ^e	899	119-36-8 	Yes Europe: 490 USA: 44000	No	Yes The NOEL of 50 mg/kg of body weight per day in a 2-year study in dogs is >100 times the estimated daily intake of methyl salicylate when used as a flavouring agent	See note 2	
Ethyl salicylate	900	118-61-6 	No Europe: 31 USA: 1700	NR	NR	See note 2	

Butyl salicylate	901	2052-14-4		No Europe: 0.01 USA: 0.0007	NR	NR	See note 2
Isobutyl salicylate	902	87-19-4		No Europe: 1 USA: 6	NR	NR	See note 2
Isoamyl salicylate	903	87-20-7		No Europe: 49 USA: 7	NR	NR	See note 2
Benzyl salicylate	904	118-58-1		No Europe: 30 USA: 29	NR	NR	See note 3
Phenethyl salicylate	905	87-22-9		No Europe: 0.2 USA: 4	NR	NR	See note 3
o-Tolyl salicylate	907	617-01-6		No Europe: ND USA: 30	NR	NR	See note 3
2,4-Dihydroxybenzoic acid	908	89-86-1		No Europe: ND USA: 6	NR	NR	See note 1

No safety concern

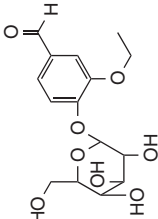
Table 5 (continued)

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Step A5 Adequate margin of safety for substance or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Structural class II							
Vanillyl ethyl ether	887	13184-86-6 	No Europe: 22 USA: 22	NR	NR	See note 1	No safety concern
Vanillyl butyl ether	888	82654-98-6 	No Europe: ND USA: 0.1	NR	NR	See note 1	
Ethyl vanillin ^f	893	121-32-4 	Yes Europe: 6200 USA: 43000	No	Yes The NOEL of 500mg/kg of body weight per day in a 14-week study in rats is >100 times the estimated daily intake of ethyl vanillin when used as a flavouring agent	See note 1	

Vanillin <i>erythro</i> - and <i>threo</i> -butan-2,3-diol acetal	960	63253-24-7		No Europe: 4 USA: 3	NR	NR	See note 2
Ethyl vanillin isobutyrate	953	188417-26-7		No Europe: 64 USA: ND	NR	NR	See note 2
Ethyl vanillin propylene glycol acetal	954	68527-76-4		No Europe: 39 USA: 36	NR	NR	See note 2
Piperonyl acetate	894	326-61-4		No Europe: 41 USA: 11	NR	NR	See note 4
Piperonyl isobutyrate	895	5461-08-5		No Europe: 0.1 USA: 3	NR	NR	See note 4
Piperonal ⁹	896	120-57-0		Yes Europe: 1700 USA: 3200	No	Yes The NOEL of 250mg/kg of body weight per day in a 2-year study in rats is >1000 times the estimated daily intake of piperonal when used as a flavouring agent	See note 4

No safety concern

Table 5 (continued)

Flavouring agent	No.	CAS number and structure	Step A3 ^a Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Step A5 Adequate margin of safety for substance or related substance?	Comments on predicted metabolism	Conclusion based on current intake
Ethyl vanillin β -D-glucopyranoside	892	122397-96-0 	No Europe: ND USA: 30	NR	NR	See note 2	No safety concern

CAS: Chemical Abstracts Service; ND: no data on intake reported; NR: not required for evaluation because consumption of the substance was determined to be of no safety concern at step A3 of the Procedure.

- ^a Step 2: All of the flavouring agents in this group are expected to be metabolized to innocuous products.
- ^b The thresholds for human intake for structural classes I and II are 1800 µg/day and 540 µg/day, respectively. All intake values are expressed in µg/day.
- ^c Further information is required to determine whether this substance is in current use as a flavouring agent.
- ^d An ADI of 0–10 mg/kg of body weight was established for vanillin by the Committee at its eleventh meeting (Annex 1, reference 14), which was maintained at the present meeting.
- ^e An ADI of 0–0.5 mg/kg of body weight was established for methyl salicylate by the Committee at its eleventh meeting (Annex 1, reference 14), which was maintained at the present meeting. The estimated daily per capita intake of methyl salicylate is 0.7 mg/kg of body weight when calculated on the basis of the usual 10% proportion of eaters; however, a survey of intake showed that >50% of the population would be expected to consume methyl salicylate. When this measured proportion of eaters was used, the calculated intake was 0.1 mg/kg of body weight.
- ^f An ADI of 0–3 mg/kg of body weight was established for ethyl vanillin by the Committee at its forty-fourth meeting (Annex 1, reference 116), which was maintained at the present meeting.
- ^g An ADI of 0–2.5 mg/kg of body weight was established for piperonal by the Committee at its eleventh meeting (Annex 1, reference 14), which was maintained at the present meeting.

Notes to Table 5

1. Detoxication by excretion in the urine unchanged or as glucuronic acid, glycine or sulfate conjugates; aldehyde groups will undergo oxidation or reduction to the corresponding carboxylic acid or alcohol, respectively, followed by conjugation and excretion; *O*-dealkylation followed by conjugation and excretion; other, minor metabolic routes, which probably occur in the intestinal microflora after biliary excretion of conjugates, include decarboxylation and reduction of benzyl groups to the methyl analogues.
2. Detoxication as described in note 1 plus hydrolysis of esters to the corresponding benzyl alcohol or benzoic acid derivatives, acetal hydrolysis to the parent benzaldehyde derivative and simple aliphatic alcohol, or glycosidic bond hydrolysis to the corresponding phenolic derivative.
3. Detoxication as described in note 1, preceded by hydrolysis to yield mononuclear residues, each of which would be detoxicated as described in note 1.
4. Detoxication as described in note 1 plus limited oxidation of the methylenedioxyphenyl group to a catechol, which would undergo conjugation.

4.1.4.1 Estimated daily per capita intake

The total annual volume of production of the 46 flavouring agents in this group is 450 tonnes in Europe and 1800 tonnes in the USA. Vanillin (No. 889), ethyl vanillin (No. 893), methyl salicylate (No. 899) and piperonal (No. 896), for which ADIs were previously established by the Committee, account for approximately 98% of the total annual volume in Europe and 99% in the USA. In Europe, the estimated daily per capita intakes of these compounds are 55 mg of vanillin, 6.2 mg of ethyl vanillin, 0.5 mg of methyl salicylate and 1.7 mg of piperonal. In the USA, the estimated daily per capita intakes are 150 mg of vanillin, 43 mg of ethyl vanillin, 44 mg of methyl salicylate and 3.2 mg of piperonal. The estimated daily per capita intakes of the other flavouring agents in this group are lower. Ethyl salicylate (No. 900) and *p*-methoxybenzaldehyde (No. 878) have the next highest daily per capita intakes; that of ethyl salicylate is 1.7 mg in the USA and that of *p*-methoxybenzaldehyde is 0.5 mg in both Europe and the USA. The remaining 40 flavouring agents have estimated daily per capita intakes of <100 µg, 10 of which are under 1 µg. The daily per capita intake of each substance in Europe and the USA is shown in Table 5.

4.1.4.2 Absorption, distribution, metabolism and elimination

The aromatic esters in this group can be expected to be hydrolysed extensively through the catalytic activity of the intestinal carboxylesterases, especially β-esterases, to benzyl alcohol or benzoic acid derivatives before absorption. Likewise, acetals of substituted benzaldehyde derivatives will be hydrolysed in gastric and intestinal fluids to yield benzaldehyde and aliphatic alcohols. The resulting hydroxy- and alkoxy-substituted benzyl derivatives are rapidly absorbed in the gut, metabolized in the liver and excreted in the urine.

Once absorbed, benzyl derivatives are oxidized to the corresponding benzoic acid derivative, which is subsequently excreted unchanged or as sulfate or glucuronide conjugates. Minor metabolic detoxication pathways include *O*-demethylation, reduction and decarboxylation. These pathways are used during enterohepatic cycling of conjugated benzyl metabolites and subsequent intestinal bacterial action. Piperonal is oxidized to piperonylic acid and excreted mainly as the glycine conjugate.

4.1.4.3 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents (see Fig. 1), the Committee assigned 36 of the 46 flavouring agents in this group to structural class I. These flavouring

agents are all either simple substituted aromatic compounds, cyclic acetals of benzaldehydes which are expected to be hydrolysed to aromatic aldehydes and simple aliphatic alcohols (Nos 870–875, 877–886, 889–891, 897–903, 908, 955–959), or compounds containing two aromatic rings which are expected to be hydrolysed to mononuclear residues with simple functional groups (Nos 876, 904, 905 and 907). The remaining 10 flavouring agents (Nos 887, 888, 892–896, 953, 954 and 960) are ethyl vanillin or piperonal derivatives that contain either an ethoxy or a methylene dioxy substituent. They are common components of food, or closely structurally related to common components of food, and were assigned to structural class II.

Step 2. At current levels of intake, the metabolic pathways of these flavouring agents can be predicted not to be saturated, and all can be predicted to be metabolized to innocuous products. The evaluation of these substances therefore proceeded down the left-hand side of the decision-tree.

Step A3. The estimated daily per capita intakes of 34 of the flavouring agents in structural class I and eight flavouring agents in structural class II were below the thresholds of concern for these classes (1800µg and 540µg, respectively). The Committee concluded that these 42 flavouring agents would not be expected to be of safety concern at currently estimated levels of use.

The estimated daily per capita intakes of vanillin (No. 889) and methyl salicylate (No. 899), which are in structural class I, exceed the threshold of concern for this class (1800µg). The estimated daily per capita intakes of vanillin are 55 000µg in Europe and 150 000µg in the USA, and that of methyl salicylate is 44 000µg in the USA. The estimated daily per capita intakes of ethyl vanillin (No. 893) and piperonal (No. 896), which are in structural class II, exceed the threshold of concern for this class (540µg). The estimated daily per capita intakes of ethyl vanillin are 6200µg in Europe and 43 000µg in the USA, and those of piperonal are 1700µg in Europe and 3200µg in the USA.

The estimated daily per capita intakes of these four substances are below their respective ADI values. The daily intakes of vanillin in Europe and the USA, approximately 0.9 and 2.5 mg/kg of body weight respectively, do not exceed the ADI of 0–10 mg/kg of body weight for vanillin. The highest estimated daily intakes of ethyl vanillin (0.7 mg/kg of body weight in the USA) and piperonal (0.05 mg/kg of body weight in the USA) do not exceed the ADIs of 0–3 mg/kg of body weight for ethyl vanillin and 0–2.5 mg/kg of body weight for piperonal. The highest estimated daily intake of the remaining substance, methyl salicylate, is 0.7 mg/kg of body weight, which is approximately equal to its ADI of 0–0.5 mg/kg of body weight.

The estimates of intake derived from total annual volume of production are based on the assumption that only 10% of the population consumes the substance under consideration. The Committee reviewed an analysis of the intake of methyl salicylate which was based on individual dietary records of consumption of baked goods, chewing-gums, hard and soft sweets and beverages in which this agent is used in the USA. The analysis showed that more than 50% of the population would be expected to consume methyl salicylate. Use of this *measured* proportion of eaters in place of the default assumption of 10% yields an estimated intake of methyl salicylate of 0.1 mg/kg of body weight, which is still below the current ADI of 0–0.5 mg/kg of body weight.

Step A4. Vanillin (No. 889), methyl salicylate (No. 899), ethyl vanillin (No. 893) and piperonal (No. 896) are not endogenous in humans. The evaluation of these substances therefore proceeded to step A5.

Step A5. The ADI of 0–10 mg/kg of body weight for vanillin is based on a NOEL of 1000 mg/kg of body weight per day in a 2-year feeding study in rats. This NOEL provides a margin of safety, as it is more than 100 times the per capita intake of vanillin from its currently estimated use as a flavouring agent in Europe (0.9 mg/kg of body weight per day) or in the USA (2.5 mg/kg of body weight per day).

The ADI of 0–0.5 mg/kg of body weight for methyl salicylate is based on a NOEL of 50 mg/kg of body weight per day reported in a 2-year study in dogs. This NOEL is more than 1000 times greater than the intake of methyl salicylate from its currently estimated use as a flavouring agent in Europe (0.008 mg/kg of body weight per day) and is more than 100 times greater than the intake of methyl salicylate in the USA when intake is calculated on the basis of the measured portion of eaters of 50% (0.1 mg/kg of body weight per day).

A NOEL of 500 mg/kg of body weight per day for ethyl vanillin was reported in a 14-week feeding study in rats. This NOEL is more than 100 times greater than the intake of ethyl vanillin from its use as a flavouring agent in Europe (0.1 mg/kg of body weight per day) or in the USA (0.7 mg/kg of body weight per day).

A NOEL of 250 mg/kg of body weight per day for piperonal was reported in a 2-year study in rats. This NOEL is more than 1000 times the intake of piperonal from its use as a flavouring agent in Europe (0.03 mg/kg of body weight per day) and in the USA (0.05 mg/kg of body weight per day).

Table 5 summarizes the evaluations of the 46 hydroxy- and alkoxy-substituted benzyl derivatives used as flavouring agents.

4.1.4.4 *Consideration of combined intakes from use as flavouring agents*

In the unlikely event that all 36 flavouring agents in structural class I were to be consumed on a daily basis, the estimated combined intake would exceed the threshold for human intake for this class (1800 µg/day). In the unlikely event that all 10 flavouring agents in structural class II were to be consumed on a daily basis, the estimated combined intake would exceed the threshold for human intake for this class (540 µg/day). However, all 46 flavouring agents in this group are expected to be efficiently detoxicated, and the available detoxication pathways would not be saturated. Evaluation of all the data indicated no safety concern associated with combined intake.

4.1.4.5 *Conclusions*

The Committee retained the previously established ADIs of 0–10 mg/kg of body weight for vanillin (No. 889), 0–3 mg/kg of body weight for ethyl vanillin (No. 893), 0–2.5 mg/kg of body weight for piperonal (No. 896) and 0–0.5 mg/kg of body weight for methyl salicylate (No. 899). The Committee noted that the estimated daily intake of 0.7 mg/kg of body weight of methyl salicylate, based on the production volume used, is approximately equal to its ADI of 0–0.5 mg/kg of body weight, within the precision of the intake estimates. The Committee reviewed an analysis of intake based on individual dietary records of consumption of mint-flavoured baked goods, chewing-gums, hard and soft sweets and beverages in which methyl salicylate is potentially used. This analysis showed that more than 50% of the population would be expected to consume methyl salicylate. The use of this *measured* proportion of eaters in place of the default assumption of 10% yields an estimated intake of methyl salicylate of 0.1 mg/kg of body weight, which is below the ADI of 0–0.5 mg/kg of body weight.

On the basis of the available data on metabolism and toxicity, the Committee concluded that the safety of the flavouring agents in this group would not raise concern at the currently estimated levels of use. Other data on toxicity, including studies on developmental toxicity and genotoxicity, were consistent with the results of the safety evaluations.

A monograph summarizing the safety data on this group of flavouring agents was prepared.

4.1.5 ***Aliphatic acyclic diols, triols and related substances***

The Committee evaluated a group of 31 flavouring agents¹ that included aliphatic acyclic diols, triols and related substances

¹ During evaluation of these flavouring agents, the Committee questioned whether some substances in this group (Nos 909 and 914–926) were used as flavouring agents and therefore appropriately evaluated by this Procedure. Information to address this question will be sought from relevant manufacturers.

(Table 6) by the Procedure for the Safety Evaluation of Flavouring Agents (see Fig. 1). All members of this group are aliphatic acyclic primary alcohols, aldehydes, acids or related esters with one or more additional oxygenated functional groups. The group comprised four subgroups: glycerol (No. 909) and 15 related glycerol esters and acetals (Nos 910–924); propylene glycol (No. 925) and four related esters, acetals and ketals (Nos 926–929); lactic acid (No. 930) and four lactate esters (Nos 931, 932, 934 and 935); and pyruvic acid (No. 936), its corresponding aldehyde (No. 937), two pyruvate esters (Nos 938 and 939) and one acetal of pyruvic acid (No. 933).

The Committee had previously evaluated three members of the group. Glycerol (No. 909) was considered at the twentieth meeting (Annex 1, reference 41), when an ADI “not specified”¹ was established. Propylene glycol (No. 925) was considered at the seventh meeting (Annex 1, reference 7), when an ADI of 0–20 mg/kg of body weight was established; it was further considered at the seventeenth meeting (Annex 1, reference 32), when the ADI was increased to 0–25 mg/kg of body weight. Ethyl lactate (No. 931) was considered at the eleventh, twenty-third, twenty-fourth and twenty-sixth meetings (Annex 1, references 14, 50, 53 and 59). At its twenty-sixth meeting, the Committee included ethyl lactate in the group ADI “not specified”¹ with lactic acid.

Nine of the 31 substances (Nos 909, 929–932, 934 and 936–938) have been detected as natural components of foods, in cocoa, milk, cider, cognac, asparagus, tomatoes and mushrooms.

4.1.5.1 Estimated daily per capita intake

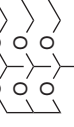
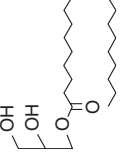
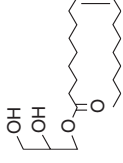
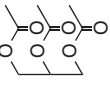
The total annual volume of production of the 31 flavouring agents in this group is 140 tonnes in Europe and 21000 tonnes in the USA. These values are equivalent to a total estimated daily per capita intake of 20000 µg in Europe and 2800000 µg in the USA. The large difference in the annual volume of production in Europe and the USA is due to the inclusion in the USA of figures on the use of glycerol, triacetin and propylene glycol as solvents in the preparation of flavour mixtures.

In Europe, 97% of the total daily per capita intake of this group of flavouring agents was accounted for by glycerol (17000 µg), ethyl lactate (1900 µg) and butyl lactate (380 µg). In the USA, 96% of the total daily per capita intake was accounted for by glycerol (220000 µg), triacetin (83000 µg) and propylene glycol (2400000 µg).

¹ See footnote on page 32.

Table 6

Summary of results of the safety evaluations of aliphatic acyclic diols, triols and related substances^a

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Conclusion based on current intake
Structural class I					
Glycerol ^{c,d}	909	56-81-5 	Yes Europe: 17000 USA: 220 000	Yes Glycerol is endogenous	Evaluation not finalized
1,2,3-Tris[(1'-ethoxy)-ethoxy]propane	913	67715-82-6 	No Europe: 0 USA: 140	NR	No safety concern
Glyceryl monostearate ^c	918	123-94-4 	No Europe: 0 USA: 230	NR	Evaluation not finalized
Glyceryl monooleate ^c	919	111-03-5 	No Europe: ND USA: 860	NR	
Triacetin ^c	920	102-76-1 	Yes Europe: ND USA: 83 000	Yes Expected to be hydrolysed to glycerol, which is endogenous	

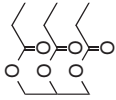
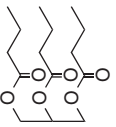
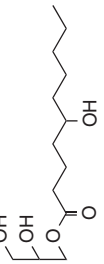
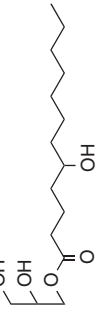
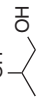
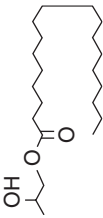
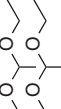
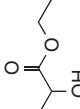
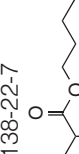
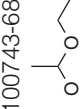
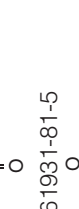
Glyceryl tripropanoate ^c	921	139-45-7 	No Europe: 0.1 USA: 280	NR	Evaluation not finalized
Tributylin ^c	922	60-01-5 	No Europe: 31 USA: 2	NR	
Glycerol 5-hydroxy-decanoate ^c	923	26446-31-1 	No Europe: 4 USA: 0	NR	
Glycerol 5-hydroxy-dodecanoate ^c	924	26446-32-2 	No Europe: 4 USA: 0	NR	
Propylene glycol ^{c,e}	925	57-55-6 	Yes Europe: ND USA: 2400000	Yes Expected to be oxidized to lactic acid, which is endogenous	
Propylene glycol stearate ^c	926	142-75-6 	Yes Europe: ND USA: 66000	Yes Expected to be hydrolysed to propylene glycol and subsequently oxidized to lactic acid	

Table 6 (continued)

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Conclusion based on current intake
1,2-Di[(1-ethoxy)- ethoxy]propane	927	67715-79-1 	No Europe: 7 USA: 150	NR	No safety concern
Lactic acid	930	598-82-3 	Yes Europe: ND USA: 47 000	Yes Lactic acid is endogenous	
Ethyl lactate ⁱ	931	97-64-3 	Yes Europe: 1900 USA: 760	Yes Expected to be hydrolysed to lactic acid, which is endogenous	
Butyl lactate	932	138-22-7 	No Europe: 380 USA: 24	NR	
Potassium 2-(1'-ethoxy)- ethoxypropanoate	933	100743-68-8 	No Europe: ND USA: 1400	NR	
cis-3-Hexenyl lactate	934	61931-81-5 	No Europe: 38 USA: 5	NR	

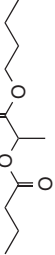
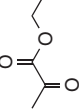
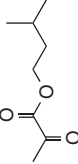
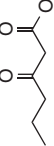
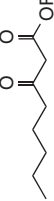
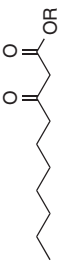
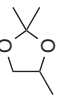
Butyl butyryllactate	935	7492-70-8		No Europe: 280 USA: 1400	NR	No safety concern
Pyruvic acid	936	127-17-3		No Europe: 35 USA: 69	NR	
Pyruvaldehyde	937	78-98-8		No Europe: 120 USA: 3	NR	
Ethyl pyruvate	938	617-35-6		No Europe: 1 USA: 20	NR	
Isoamyl pyruvate	939	7779-72-8		No Europe: 0 USA: 0	NR	
Structural class III 3-Oxohexanoic acid glyceride	910	91052-72-1		Yes Europe: 0 USA: 270	Yes Expected to be hydrolysed to glycerol, which is endogenous NR	No safety concern
3-Oxooctanoic acid glyceride	911	91052-68-5		No Europe: 34 USA: 0	NR	
Heptanal glyceryl acetal (mixed 1,2 and 1,3 acetals)	912	1708-35-6		No Europe: 4 USA: 0	NR	

Table 6 (continued)

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Step A4 Is the substance or are its metabolites endogenous?	Conclusion based on current intake
3-Oxodecanoic acid glyceride ^c	914	91052-69-6 	Yes Europe: 0 USA: 270	Yes Expected to be hydrolysed to glycerol, which is endogenous NR	Evaluation not finalized
3-Oxododecanoic acid glyceride ^c	915	91052-70-9 	No Europe: 73 USA: 0		
3-Oxotetradecanoic acid glyceride ^c	916	91052-73-2 	Yes Europe: 0 USA: 270	Yes Expected to be hydrolysed to glycerol, which is endogenous NR	
3-Oxohexadecanoic acid glyceride ^c	917	91052-71-0 	No Europe: 43 USA: 0		No safety concern
4-Methyl-2-pentyl-1,3-dioxolane	928	26563-74-6 	No Europe: 0 USA: 0.2	NR	
2,2,4-Trimethyl-1,3-oxacyclopentane	929	1193-11-9 	No Europe: 0.3 USA: 0.2	NR	

CAS: Chemical Abstracts Service; ND: no data on intake reported; NR: not required for evaluation because consumption of the substance was determined to be of no safety concern at step A3 of the Procedure.

^a Step 2: All of the flavouring agents in this group are predicted to be metabolized to innocuous products.

^b The threshold for human intake for structural classes I and III are 1800 µg/day and 90 µg/day, respectively. All intake values are expressed in µg/day.

^c Further information is required to determine whether this substance is in current use as a flavouring agent.

^d An ADI "not specified" was established for glycerol by the Committee at its twentieth meeting (Annex 1, reference 41), which was maintained at the present meeting.

^e An ADI of 0–25 mg/kg of body weight was established for propylene glycol by the Committee at its seventeenth meeting (Annex 1, reference 32), which was maintained at the present meeting.

^f Ethyl lactate was included in the group ADI "not specified" for lactic acid and its salts that was established by the Committee at its twenty-sixth meeting (Annex 1, reference 59), which was maintained at the present meeting.

The daily per capita intake of each substance in Europe and the USA is shown in Table 6.

4.1.5.2 Absorption, distribution, metabolism and elimination

The aliphatic esters of propylene glycol (No. 925), lactic acid (No. 930) and pyruvic acid (No. 936) and their parent compounds would all be expected to be readily absorbed from the gut. Hydrolysis of the aliphatic esters is catalysed largely by hepatic esterases, to give the component alcohol and carboxylic acid or aldehyde. After hydrolysis of the glycerol esters in the intestine, glycerol is readily absorbed. Glycerol, pyruvic acid and lactic acid are endogenous in humans. Glycerol and pyruvic acid are metabolized completely and are not excreted. Lactic acid is also largely metabolized, although urinary excretion may occur if the blood concentration is high. Propylene glycol can be metabolized, but high doses are likely to be excreted largely unchanged in the urine.

Glycerol (No. 909) is converted in the liver to glycerol-3-phosphate, which is metabolized via the glycolytic pathway, by oxidation, to yield dihydroxyacetone phosphate, which is isomerized to glyceraldehyde-3-phosphate, eventually yielding pyruvic acid.

Pyruvic acid follows two primary routes of metabolism. Under aerobic conditions, it is converted to acetyl coenzyme A and enters the citric acid cycle. Under anaerobic conditions, primarily in muscles as a result of strenuous physical activity, pyruvic acid is reduced by lactic dehydrogenase to lactic acid.

Lactic acid diffuses through muscle tissue and is transported to the liver in the bloodstream. In the liver, it is converted to glucose by gluconeogenesis. Lactic acid can also be further catabolized in the lactic acid cycle (also known as the Cori cycle).

Propylene glycol can be oxidized to lactic acid via two biochemical pathways. If propylene glycol is phosphorylated, it can be converted to acetol phosphate, lactaldehyde phosphate, lactyl phosphate and then lactic acid. If it is not phosphorylated, propylene glycol is successively oxidized to lactaldehyde, methylglyoxal and lactic acid.

4.1.5.3 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. Twenty-eight of the 31 flavouring agents in this group are linear, simple-branched aliphatic compounds. In applying the Procedure for the Safety Evaluation of Flavouring Agents (see Fig. 1), the Committee assigned 22 of the agents to structural class I because they contain fewer than three types of functional group (Nos 909, 913,

918–927 and 930–939). The Committee assigned six of the substances to structural class III because they contain three or more types of functional group (Nos 910, 911 and 914–917). The three remaining substances were also assigned to structural class III because they are cyclic acetals and ketals (Nos 912, 928 and 929).

Step 2. The data on the metabolism of individual members of the group were sufficient to allow conclusions about their probable metabolic fate. The aliphatic esters of propylene glycol (No. 926), lactic acid (Nos 931, 932, 934 and 935) and pyruvic acid (Nos 938 and 939) can be expected to be hydrolysed to their component alcohols and carboxylic acids. The glycerol esters (Nos 910, 911 and 914–924) can be expected to be hydrolysed to glycerol and carboxylic acids. Esters of propylene glycol can be expected to be hydrolysed to propylene glycol and its component acid. Esters of lactic acid and pyruvic acid can be expected to be hydrolysed to lactic acid and pyruvic acid, respectively, and the corresponding alcohols. The acetals (Nos 912, 913, 927 and 933) can be expected to be hydrolysed to their component alcohols and aldehydes, while the ketals (Nos 928 and 929) can be expected to be hydrolysed to their component ketones and alcohols. Glycerol (No. 909), lactic acid (No. 930) and pyruvic acid (No. 936) are endogeneous and are metabolized through the glycolytic and citric acid pathways. Propylene glycol (No. 925) is oxidized to lactic acid. Because they are predicted to be metabolized to innocuous products, the evaluation of all the substances in this group proceeded via the left-hand side of the decision-tree.

Step A3. The estimated daily per capita intakes of 17 of the substances in structural class I and five substances in structural class III are below the threshold of concern for these classes (1800 µg and 90 µg, respectively). The Committee concluded that these substances would not be expected to be of safety concern at their currently estimated levels of use. The daily per capita intakes of six substances in structural class I (Nos 909, 920, 925, 926, 930 and 931) and three substances in structural class III (Nos 910, 914 and 916) exceed the threshold of concern for these classes, and their evaluation therefore proceeded to step A4.

Step A4. Glycerol (No. 909), lactic acid (No. 930) and the hydrolysis products of ethyl lactate (No. 931) are endogenous in humans and their use as flavouring agents is therefore not expected to be of safety concern. Triacetin (No. 920), 3-oxohexanoic acid glyceride (No. 910), 3-oxodecanoic acid glyceride (No. 914) and 3-oxotetradecanoic acid glyceride (No. 916) are glycerol esters and are hydrolysed to glycerol.

Propylene glycol (No. 925) and propylene glycol stearate (No. 926) are not endogenous in humans; however, the ester is expected to be hydrolysed to propylene glycol and stearic acid. Propylene glycol is known to be oxidized to lactic acid in mammals. The safety of these substances would therefore not be expected to be of concern.

4.1.5.4 Consideration of combined intakes from use as flavouring agents

In the unlikely event that all 23 substances in structural class I were to be consumed concurrently on a daily basis, the estimated combined intake would exceed the threshold for human intake for this class (1800µg). In the unlikely event that all eight substances in structural class III were to be consumed concurrently on a daily basis, the estimated combined intake would exceed the threshold for human intake for this class (90µg). Given that the substances can be expected to be efficiently metabolized by known metabolic pathways, the Committee considered that the combined intake would not give rise to concern about safety.

4.1.5.5 Conclusions

On the basis of the predicted metabolism, the Committee concluded that the safety of the 31 aliphatic acyclic diols, triols and related substances in this group would not raise concern at the currently estimated levels of use as flavouring agents. The Committee noted that all of the available data on toxicity were consistent with the results of the safety evaluations.

A monograph summarizing the safety data on this group of flavouring agents was prepared.

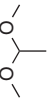
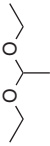
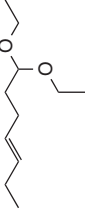
4.1.6 Aliphatic acyclic acetals

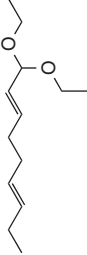
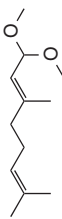
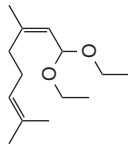
The Committee evaluated a group of 10 flavouring agents consisting of aliphatic acyclic acetals (Table 7) by the Procedure for the Safety Evaluation of Flavouring Agents (see Fig. 1). They all have acyclic structures that vary only in the length of their hydrocarbon chains and the number and placement of double bonds. None of these flavouring agents had been evaluated previously by the Committee.

Aliphatic acetals are geminal diethers in which two molar equivalents of alcohol are condensed with an aldehyde. Three of the 10 acetals in this group are formed from acetaldehyde and simple aliphatic alcohols (Nos 940, 941 and 943); the remaining seven acetals are formed from methanol or ethanol and aldehydes of carbon chain-length C7–C10 (Nos 942 and 944–949). Acetals are known to be hydrolysed *in vivo* to yield the corresponding alcohols and aldehydes. Of the component alcohols (methanol, ethanol and *cis*-3-hexen-1-ol)

Table 7

Summary of results of the safety evaluations of aliphatic acyclic acetals used as flavouring agents^a

Flavouring agent	No.	CAS number and structure	Step A3 ^b Does intake exceed the threshold for human intake?	Comments on predicted metabolism	Conclusion based on current intake
1,1-Dimethoxyethane	940	534-15-6 	No Europe: 71 USA: 11	Metabolized to acetaldehyde and methanol	No safety concern
Acetal	941	105-57-7 	No Europe: 240 USA: 640	Metabolized to acetaldehyde and ethanol	
Heptanal dimethyl acetal	947	10032-05-0 	No Europe: 0.04 USA: 0.26	Metabolized to heptanal and methanol	
4-Heptenal diethyl acetal	949	18492-65-4 	No Europe: 0.04 USA: 0	Metabolized to 4-heptenal and ethanol	
Octanal dimethyl acetal	942	10022-28-3 	No Europe: 1.1 USA: 0	Metabolized to octanal and methanol	

2,6-Nonadienal diethyl acetal	946	67674-36-6		No Europe: 0.04 USA: 0.01	Metabolized to 2,6-nonadienal and ethanol
Decanal dimethyl acetal	945	7779-41-1		No Europe: 0.03 USA: 0	Metabolized to decanal and methanol
Citral dimethyl acetal	944	7549-37-3		No Europe: 3 USA: 5	Metabolized to citral and methanol
Citral diethyl acetal	948	7492-66-2		No Europe: 4 USA: 0	Metabolized to citral and ethanol
Acetaldehyde ethyl cis-3-hexenyl acetal	943	28069-74-1		No Europe: ND USA: 0	Metabolized to acetaldehyde, ethanol and cis-3-hexen-1-ol

No safety concern

CAS: Chemical Abstracts Service; ND: no data on intake reported.

^a Step 1: All of the flavouring agents in this group are in structural class I.

^b Step 2: All of the flavouring agents in this group are expected to be metabolized to innocuous products.

The threshold for human intake for structural class I is 1800 µg/day. All intake values are expressed in µg/day.

and aldehydes, acetaldehyde, heptanal, 4-heptenal, octanal and decanal had been considered previously by the Committee at its forty-ninth and fifty-first meetings (Annex 1, references 131 and 137), when it concluded that their safety was of no concern at currently estimated levels of use as flavouring agents.

Three of the 10 flavouring agents in this group, 1,1-dimethoxyethane (No. 940), acetal (No. 941) and acetaldehyde ethyl *cis*-3-hexenyl acetal (No. 943), have been reported to occur as natural components of foods. They have been detected in orange juice, strawberries, cider, peas, coffee and cognac.

4.1.6.1 Estimated daily per capita intake

The total annual volume of production of the 10 aliphatic acyclic acetals is approximately 2.2 tonnes in Europe and 4.9 tonnes in the USA. About 97% of the total annual volume of production in Europe and 99% of that in the USA is accounted for by 1,1-dimethoxyethane (No. 940), formed from acetaldehyde and methanol, and acetal (No. 941), formed from acetaldehyde and ethanol.

4.1.6.2 Absorption, distribution, metabolism and elimination

In general, aliphatic acetals undergo acid-catalysed hydrolysis to their component aldehydes and alcohols. They are hydrolysed within 1–5 h in simulated gastric fluid *in vitro* and to a lesser extent in simulated intestinal fluid. Indirect evidence, from a study in which rabbits were given aliphatic acetals in aqueous suspension by stomach tube, indicated that rapid hydrolysis occurs in the stomach. The acetals formed from the reaction of alkyl-substituted pentanal with methanol, ethanol and isopropyl alcohol are metabolized to the corresponding alcohols and acids in rat liver homogenates by an oxidative mechanism involving cytochrome P450 enzymes. Aliphatic acetals can be expected to undergo similar metabolism in humans to the corresponding alcohols and acids. There are insufficient data to exclude the possibility that significant amounts of the parent acetals reach the general circulation; however, the parent compounds are all in structural class I. The low intake resulting from uses of these substances as flavours would not be expected to saturate metabolic enzyme pathways, and the acetals are metabolized to innocuous compounds by hydrolysis or oxidation.

On the basis of their recognized or presumed metabolic fate, the component alcohols and aldehydes can be grouped into three structural classes: linear, aliphatic, primary, saturated and unsaturated alcohols and aldehydes; α,β -unsaturated aldehydes; and branched-chain aliphatic aldehydes. The metabolic detoxication of linear, ali-

phatic, primary alcohols in vivo occurs primarily by oxidation to the corresponding aldehyde, with subsequent oxidation of the aldehyde to the corresponding carboxylic acid. The acid can serve as a substrate for fatty acid oxidation pathways and the citric acid cycle. In general, α,β -unsaturated aldehydes are metabolized by oxidation to the corresponding carboxylic acid, which may then participate in the fatty acid pathway. The aldehyde can also be conjugated with glutathione in a Michael-type addition. Branched-chain aliphatic aldehydes are oxidized primarily to polar metabolites, which are excreted mainly in the urine. The main urinary metabolites of branched aldehydes are diacids and hydroxyacids resulting from ω -oxidation, reduction and hydration of the alkene function and oxidation of the aldehyde function.

Although few studies on the absorption, distribution and elimination of aliphatic acyclic acetals have been reported, the metabolism of the component alcohols and aldehydes has been investigated. These studies are considered relevant to the safety evaluation of orally administered acetals which are expected to be hydrolysed in the acid environment of the stomach.

Citral is predicted to be a metabolite of citral dimethyl acetal (No. 944) and citral diethyl acetal (No. 948). The absorption, distribution and excretion of citral have been studied extensively in rats and mice. Citral underwent rapid absorption from the gut and was distributed uniformly throughout the body. Rapid elimination of citral and its metabolites occurred primarily in the urine and to a minor extent in exhaled air and faeces.

4.1.6.3 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure for the Safety Evaluation of Flavouring Agents (see Fig. 1), the Committee assigned all 10 of the aliphatic acetals to structural class I.

Step 2. At current levels of intake, all of these flavouring agents can be predicted to be metabolized to their component aldehydes and alcohols, which are then metabolized to innocuous products,¹ and the pathways involved would not be expected to be saturated. Evaluation of these substances therefore proceeded via the left-hand side of the decision-tree.

¹ Some aldehydes, including acetaldehyde, were genotoxic in vitro in a number of test systems, and acetaldehyde has been reported to produce tumours of the respiratory tract in rats and hamsters exposed to high doses by inhalation. The relevance of this observation to oral administration is questionable, as various metabolic processes in the intestinal wall and liver (i.e. oxidation and conjugation) are predicted to result in extensive first-pass metabolic inactivation, especially at the low concentrations expected from use of these substances as flavours.

Step A3. The estimated daily per capita intakes of all 10 substances in this group are below the threshold of concern for structural class I (1800µg). The Committee concluded that their safety raises no concern at their currently estimated levels of use as flavouring agents.

Table 7 summarizes the results of the evaluations of the aliphatic acetals used as flavouring agents.

4.1.6.4 Consideration of combined intakes from use as flavouring agents

In the unlikely event that all 10 aliphatic acetals were consumed concurrently on a daily basis, the estimated combined intake would not exceed the threshold for human intake for structural class I (1800µg/day). All flavouring agents in this group are expected to be efficiently metabolized and the available metabolic pathways would not be saturated. Evaluation of all the data indicated there would be no safety concern associated with combined intake.

4.1.6.5 Conclusions

The Committee concluded that the safety of aliphatic acetals would not raise concern at the currently estimated levels of intake. Other data on the toxicity of aliphatic acetals were consistent with the results of the safety evaluation.

A monograph summarizing the safety data on this group of flavouring agents was prepared.

4.2 Revision of certain specifications for purity

4.2.1 *Flavouring agents with specifications designated as “tentative” at previous meetings*

At its forty-sixth, forty-ninth, fifty-first, fifty-third and fifty-fifth meetings (Annex 1, references 122, 131, 137, 143 and 149), the Committee evaluated a total of 143 flavouring agents for which further information was required in order to complete the specifications. At its present meeting, the Committee reviewed new data on 95 of these flavouring agents and revised the specifications to take account of the new information. For 83 substances, the “tentative” designation was removed; for the remaining 12, the revised specifications were classified as “tentative” (see Annex 2).

For the remaining 47 substances, no further data were provided that would permit the preparation of satisfactory specifications, according to the criteria identified by the Committee at its fifty-third meeting (Annex 1, reference 143). The flavour industry has indicated that many of these substances are highly noxious, sulfur-containing compounds which are not routinely handled in the pure form. It is there-

fore impracticable to provide information on, for instance, boiling-point or specific gravity. The Committee concluded that, in such cases, the additional criteria identified at its fifty-third meeting for the establishment of satisfactory specifications should not apply and that only the basic data on chemical identity, minimum assay and identity test were required.

The Committee reiterated that the evaluation of flavouring agents relies on adequate information about the identity and composition of products in commercial use. In future, specifications for flavouring agents will be withdrawn within 2 years of publication of tentative specifications, if the necessary information is not provided.

4.2.2 *Flavouring agents with minimum assay values less than 95%*

At its fifty-third meeting (Annex 1, reference 143), the Committee announced its intention to re-examine specifications for flavouring agents for which the minimum assay values were <95% (see also section 2.6 of the present report), which were designated as “tentative”. At its present meeting, the Committee considered information on all 62 remaining substances in this category with regard to the secondary components that might be present in commercial products.

The available information was sufficient for the Committee to revise the specifications for 27 flavouring agents (including two flavouring agents for which the specifications were revised for other reasons). The Committee therefore removed the “tentative” designations, on the basis of the general principle that no more than 5% of any commercial product should remain undefined after taking account of the flavouring agent and named secondary components.

The Committee will review this group of flavouring agents at a future meeting to confirm whether all the named secondary components are covered by existing safety evaluations. At the same time, the Committee will determine the need for further information to complete those specifications that remain tentative.

4.2.3 *Specifications for flavouring agents being reviewed for safety*

A total of 203 flavouring agents were reviewed for safety at the present meeting. For four of them, specifications had been drawn up at the forty-sixth meeting (Annex 1, reference 122), and these were revised for use of these substances as flavouring agents. For six flavouring agents, information on specifications was not submitted. These will be considered at a future meeting when such data become available.

New specifications were drawn up for the remaining 193 flavouring agents. For 27 substances, the new specifications were designated as “tentative”, either because they failed to meet the criteria drawn up at the fifty-third meeting (Annex 1, reference 143) or because certain aspects required clarification.

5. Contaminants

5.1 Chloropropanols

Certain chlorinated propanols occur as contaminants in hydrolysed vegetable proteins. Processing of defatted vegetable proteins by traditional hydrochloric acid hydrolysis leads to the formation of 3-chloro-1,2-propanediol and 1,3-dichloro-2-propanol. These two substances were evaluated by the Committee at its forty-first meeting (Annex 1, reference 107), when it concluded that they are undesirable contaminants in food and expressed the opinion that their concentrations in hydrolysed vegetable proteins should be reduced as far as is technically achievable. The present evaluations were conducted in response to a request by the Codex Committee on Food Additives and Contaminants at its Thirty-second Session (4) for the Expert Committee to re-evaluate 3-chloro-1,2-propanediol and 1,3-dichloro-2-propanol on the basis of new data that had become available since the forty-first meeting.

5.1.1 **3-Chloro-1,2-propanediol**

5.1.1.1 Absorption, distribution, metabolism and excretion

3-Chloro-1,2-propanediol crosses the blood–testis barrier and the blood–brain barrier and is widely distributed in the body fluids. The parent compound is partly detoxified by conjugation with glutathione, resulting in excretion of the corresponding mercapturic acid, and is partly oxidized to β -chlorolactic acid and further to oxalic acid. Approximately 30% is broken down to carbon dioxide and exhaled. In the studies from which these data were derived, however, much of the administered dose was not accounted for. Intermediate formation of an epoxide has been postulated but not proven. There is some indication that microbial enzymes can dehalogenate halogenated alcohols to produce glycidol, a known genotoxin in vitro and in vivo.

5.1.1.2 Toxicological studies

The median lethal dose of 3-chloro-1,2-propanediol in rats after oral administration was 150mg/kg of body weight.

In several studies in which 3-chloro-1,2-propanediol was administered orally to rats as repeated doses of >1 mg/kg of body weight per day, it

decreased sperm motility and impaired male fertility. At doses of ≥ 10 – 20 mg/kg of body weight per day, alterations in sperm morphology and epididymal lesions (spermatocoele) were found. The compound reduced fertility in males of several other mammalian species at slightly higher doses than in the rat.

In rats and mice, oral administration of 3-chloro-1,2-propanediol at doses of ≥ 25 mg/kg of body weight per day was associated with the development of dose-related lesions of the central nervous system, particularly in the brain stem.

In several short-term studies in rats and mice, the kidney was shown to be the target organ for toxicity. In a 4-week study in rats treated by gavage at 30 mg/kg of body weight per day and in a 13-week study in rats given an oral dose of 9 mg/kg of body weight per day, 3-chloro-1,2-propanediol increased the weight of the kidneys relative to body weight.

In the pivotal long-term study in Fischer 344 rats, the absolute weight of the kidney was reported to be significantly increased by administration of 3-chloro-1,2-propanediol in drinking-water, at all doses tested. The incidence of tubule hyperplasia in the kidneys of treated animals of both sexes was also higher than in controls. Although the incidence did not reach statistical significance at the lowest dose tested (1.1 mg/kg of body weight per day), the Committee concluded that it represented part of a compound-related dose–response relationship. Overt nephrotoxicity was seen at higher doses (5.2 and 28 mg/kg of body weight per day).

The results of most assays for mutagenicity in bacteria *in vitro* were reported to be positive, although negative results were obtained in the presence of an exogenous metabolic activation system from mammalian tissue. The results of assays in mammalian cells *in vitro* were also generally positive. It should be noted, however, that the concentrations used in all these assays were very high (0.1–9 mg/ml), so that their relevance might be questionable. The weight of the evidence indicates that 3-chloro-1,2-propanediol is not genotoxic *in vitro* at concentrations at which other toxic effects are not observed. The results of assays conducted *in vivo*, including a test for micronucleus formation in mouse bone marrow and an assay for unscheduled DNA synthesis in rats, were negative. The Committee concluded that 3-chloro-1,2-propanediol is not genotoxic *in vivo*.

Four long-term studies of toxicity and carcinogenicity were available. Three (two in mice and one in rats) did not meet modern standards of quality; nevertheless, none of these three studies indicated

carcinogenic activity. In the fourth study, conducted in Fischer 344 rats, oral administration of 3-chloro-1,2-propanediol was associated with increased incidences of benign tumours in some organs. These tumours occurred only at doses greater than those causing renal tubule hyperplasia, which had been selected as the most sensitive end-point.

5.1.1.3 Occurrence

3-Chloro-1,2-propanediol has been detected at concentrations >1 mg/kg in only two food ingredients: acid-hydrolysed vegetable protein and soya sauce. In both ingredients, a range of concentrations has been reported, from below the limit of quantification (0.01 mg/kg) with an analytical method that has been validated in various foods and food ingredients, up to 100 mg/kg in some samples of acid-hydrolysed vegetable protein and more than 300 mg/kg in some samples of soya sauce.

Formation of 3-chloro-1,2-propanediol in acid-hydrolysed vegetable protein has been found to be related to production processes, and the concentration can be reduced markedly by modifying the processes suitably. The source of 3-chloro-1,2-propanediol in soya sauce is being investigated; by analogy with hydrolysed vegetable protein, however, it may arise during acid hydrolysis in the manufacture of some products. Traditionally fermented soya sauces would not be contaminated with 3-chloro-1,2-propanediol.

3-Chloro-1,2-propanediol has also been quantified at concentrations generally <0.1 mg/kg in other foods and food ingredients, notably a number of cereal products that have been subjected to high temperatures during roasting or toasting. Concentrations ≤ 0.5 mg/kg have been found in food ingredients such as malt extracts, but the resulting concentrations in finished foods were <0.01 mg/kg.

5.1.1.4 Estimates of dietary intake

Information on the concentrations of 3-chloro-1,2-propanediol in foods, food ingredients and protein hydrolysates was submitted by the United Kingdom and the USA and by the International Hydrolyzed Protein Council. The USA supplied a national estimate of the intake of 3-chloro-1,2-propanediol, and information on the consumption of soya sauce in Australia, Japan and the USA was received.

The toxicological studies summarized above indicate that 3-chloro-1,2-propanediol would not be expected to have acute effects at any level of intake that might reasonably be expected. This analysis therefore addressed only long-term intake of 3-chloro-1,2-propanediol from foods.

Intake of 3-chloro-1,2-propanediol would be due predominantly to consumption of contaminated soya sauces. In a survey of 90 samples of commercial soya sauces, 50 samples contained <1 mg/kg, and the average concentration was 18 mg/kg. The results of this survey were taken as representative for all soya sauces for the purposes of the intake assessment.

The mean daily per capita consumption of soya sauce in Australia by persons consuming this product was about 11 g, and that of persons at the 95th percentile of consumption was about 35 g. Thus, the daily per capita intake of 3-chloro-1,2-propanediol would be 200 µg for mean consumption of soya sauce and 630 µg at the 90th percentile of consumption. The estimated mean daily per capita consumption of soya sauce in Japan (equivalent to consumption by consumers only, in view of its widespread use in that country) was about 30 g, resulting in a mean daily per capita intake of 3-chloro-1,2-propanediol of about 540 µg. Intake at the 95th percentile in Japan was estimated to be 1100 µg by assuming twice the mean consumption of soya sauce. The estimated mean daily per capita consumption of soya sauce in the USA by consumers of this product was 8 g, and that of consumers at the 90th percentile of consumption was 16 g. The resulting estimated daily per capita intake of 3-chloro-1,2-propanediol was 140 µg for mean consumption and 290 µg for consumption at the 90th percentile.

The data submitted by the United Kingdom showed that 3-chloro-1,2-propanediol occurs in some savoury foods, with about 30% of samples containing concentrations above the limit of detection of 0.01 mg/kg. The mean residual concentration in these savoury foods was 0.012 mg/kg.

In estimating the intake of 3-chloro-1,2-propanediol from foods other than soya sauce, the Committee assumed that about one-eighth of the diet, i.e. 180 g (on the basis of 1500 g/day of solid food), consists of savoury foods that might contain 3-chloro-1,2-propanediol and that the mean residual concentration of the compound in those foods is 0.012 mg/kg. On this basis, the daily per capita intake of 3-chloro-1,2-propanediol from foods other than soya sauce was estimated to be 2 µg.

5.1.1.5 Evaluation

The Committee chose tubule hyperplasia in the kidney as the most sensitive end-point for deriving a tolerable intake. This effect was seen in the long-term study of toxicity and carcinogenicity in rats in a dose-related manner, although the effect did not reach statistical significance at the lowest dose. The Committee concluded that the

lowest-observed-effect level (LOEL) was 1.1 mg/kg of body weight per day and considered this to be close to a NOEL.

The Committee established a provisional maximum tolerable daily intake (PMTDI) of 2 µg/kg of body weight for 3-chloro-1,2-propanediol on the basis of the LOEL of 1.1 mg/kg of body weight per day and a safety factor of 500, which included a factor of 5 for extrapolation from a LOEL to a NOEL. This factor was considered to be adequate to allow for the absence of a clear NOEL and to account for the effects on male fertility and for inadequacies in the studies of reproductive toxicity. Data available to the Committee indicated that the estimated mean intake of 3-chloro-1,2-propanediol by consumers of soya sauce would be at or above this PMTDI.

5.1.1.6 Impact of regulatory limits

As 3-chloro-1,2-propanediol is found infrequently in foods, a regulatory limit would be unlikely to have much effect on the overall intake by persons who do not consume soya sauce. However, because the distribution of residual 3-chloro-1,2-propanediol in soya sauce is highly skewed and because it is likely that brand loyalty could result in regular consumption of highly contaminated brands of soya sauce, a regulatory limit on the concentration of 3-chloro-1,2-propanediol in soya sauce could markedly reduce the intake by consumers of this commodity.

5.1.2 1,3-Dichloro-2-propanol

5.1.2.1 Absorption, distribution, metabolism and excretion

Approximately 5% of an oral dose of 1,3-dichloro-2-propanol was excreted in the urine of rats as β -chlorolactate and about 1% of the dose as 2-propanol-1,3-dimercapturic acid. In another experiment, the urine of rats contained the parent compound (2.4% of the dose), 3-chloro-1,2-propanediol (0.35% of the dose) and 1,2-propanediol (0.43% of the dose). Epoxy-chloropropane (epichlorohydrin) was postulated to be an intermediate, and this compound may either undergo conjugation with glutathione to form mercapturic acid or be hydrolysed to 3-chloro-1,2-propanediol. The latter undergoes oxidation to β -chlorolactate, which is further oxidized to oxalic acid.

5.1.2.2 Toxicological studies

The median lethal dose of 1,3-dichloro-2-propanol in rats treated orally was 120–140 mg/kg of body weight.

In several short-term studies in rats, oral administration of 1,3-dichloro-2-propanol at doses of ≥ 10 mg/kg of body weight per day

caused significant hepatic toxicity. This was associated with oxidative metabolism, which yielded intermediates that reacted with and depleted glutathione.

In a 13-week study in rats, overt hepatotoxicity, including increased liver weights, histological changes and/or increased activity of serum alanine and aspartate transaminases, was seen after oral administration of 1,3-dichloro-2-propanol at doses of ≥ 10 mg/kg of body weight per day. These doses also caused histopathological changes in the kidney, increased kidney weights and alterations in urinary parameters. The NOEL was 1 mg/kg of body weight per day.

1,3-Dichloro-2-propanol has been reported to be hepatotoxic in humans exposed occupationally.

1,3-Dichloro-2-propanol was clearly mutagenic and genotoxic in various bacterial and mammalian test systems *in vitro*. The only available study *in vivo* showed no mutagenic effect in a wing spot test in *Drosophila melanogaster*.

The results of the one long-term study of toxicity and carcinogenicity in rats confirmed the hepatotoxicity and nephrotoxicity seen in the 13-week study. Furthermore, it demonstrated a clear carcinogenic effect of 1,3-dichloro-2-propanol at the highest dose tested, 19 mg/kg of body weight per day. The tumours (adenomas and carcinomas) occurred in liver, kidney, the oral epithelium and tongue and the thyroid gland. No increase in tumour incidence was seen at the lowest dose tested, 2.1 mg/kg of body weight per day. Treatment-related non-neoplastic lesions of the liver were observed, sinusoidal peliosis being found in all treated groups.

5.1.2.3 Occurrence

Information on the concentrations of 1,3-dichloro-2-propanol in soya sauce was submitted by the USA. Additional information was derived from a published report on the concomitant occurrence of 3-chloro-1,2-propanediol and 1,3-dichloro-2-propanol in soya sauces, which showed that 1,3-dichloro-2-propanol may be present at concentrations >1 mg/kg in samples of hydrolysed vegetable protein and soya sauce that contain 3-chloro-1,2-propanediol. In those products in which 1,3-dichloro-2-propanol was quantifiable, the ratio of the concentrations of 3-chloro-1,2-propanediol and 1,3-dichloro-2-propanol was at least 20.

5.1.2.4 Estimates of dietary intake

A report from the USA was used by the Committee to estimate the intake of 1,3-dichloro-2-propanol present in soya sauces. Information

about the consumption of soya sauce was received from Australia, Japan and the USA.

The toxicological studies summarized above indicate that 1,3-dichloro-2-propanol would not be expected to have acute effects at any level of intake that might reasonably be expected. This analysis therefore addressed only long-term intake of the compound from foods.

The upper-bound 20:1 ratio of 3-chloro-1,2-propanediol:1,3-dichloro-2-propanol was used by the Committee to estimate the intake of 1,3-dichloro-2-propanol from consumption of soya sauce. As the average concentration of 3-chloro-1,2-propanediol in a survey of 90 commercially obtained soya sauce samples was 18mg/kg, the residual concentration of 1,3-dichloro-2-propanol was assumed to be 0.9mg/kg.

The mean daily per capita consumption of soya sauce in Australia was approximately 11g, and that of persons at the 95th percentile of consumption was 35g. The estimated daily per capita intake was therefore 10µg for consumers at the mean and 30µg at the 95th percentile. The daily per capita intake of soya sauce in Japan (equivalent to the consumption by consumers only, in view of its widespread use in that country) was 30g, resulting in an estimated daily per capita intake of 1,3-dichloro-2-propanol of 27µg. An intake of 55µg/person per day was estimated for consumers in the upper percentile of consumption by assuming twice the mean consumption of soya sauce. The estimated mean daily per capita consumption of soya sauce in the USA by consumers of this product was 8g, and that of consumers at the 90th percentile of consumption was 16g. The resulting estimated daily per capita intake of 1,3-dichloro-2-propanol was 7µg at the mean level of consumption and 14µg at the 90th percentile.

The intake of 1,3-dichloro-2-propanol from foods other than soya sauce can be estimated roughly from data on residual concentrations of 3-chloro-1,2-propanediol in savoury foods and the upper-bound 20:1 ratio of 3-chloro-1,2-propanediol:1,3-dichloro-2-propanol. If it is assumed that about one-eighth of the diet, i.e. 180g (on the basis of 1500g/day of solid food), consists of savoury foods that might contain 1,3-dichloro-2-propanol and that the mean residual concentration of the compound in those foods is 0.6µg/kg, the background daily per capita intake is approximately 0.1µg.

5.1.2.5 Evaluation

Although only a few studies of kinetics, metabolism, short- and long-term toxicity and reproductive toxicity were available for evaluation,

the results clearly indicated that 1,3-dichloro-2-propanol was genotoxic in vitro, was hepatotoxic and induced a variety of tumours in various organs in rats. The Committee concluded that it would be inappropriate to estimate a tolerable intake because of the nature of the toxicity observed:

- The results of the long-term study of toxicity and carcinogenicity showed significant increases in the incidences of both benign and malignant neoplasms in at least three different tissues.
- It has been shown unequivocally that this contaminant can interact with chromosomes and/or DNA; however, the tests were confined to bacterial and mammalian test systems in vitro, and there were no data on intact mammalian organisms or humans.

The Committee noted that the dose that caused tumours in rats (19 mg/kg of body weight per day) was about 20 000 times the highest estimated intake of 1,3-dichloro-2-propanol by consumers of soya sauce (1 µg/kg of body weight per day).

The available evidence suggests that 1,3-dichloro-2-propanol is associated with high concentrations of 3-chloro-1,2-propandiol in food. Regulatory control of the latter would therefore obviate the need for specific controls on 1,3-dichloro-2-propanol.

5.2 Polychlorinated dibenzodioxins, polychlorinated dibenzofurans and coplanar polychlorinated biphenyls

5.2.1 Introduction

Polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are by-products of combustion and of various industrial processes, and they are found widely in the environment. Polychlorinated biphenyls (PCBs) were manufactured in the past for a variety of industrial uses, notably as electrical insulators or dielectric fluids and specialized hydraulic fluids. Most countries banned manufacture and use of PCBs in the 1970s; however, past improper handling of PCBs constitutes a continuing source of these compounds in the environment, and disposal of equipment containing these compounds poses some risk of further contamination.

Neither PCDDs nor PCDFs have been evaluated previously by the Committee. PCBs were evaluated by the Committee at its thirty-fifth meeting (Annex 1, reference 88), when it concluded that it was impossible to establish a precise numerical value for a tolerable intake in humans because of limitations in the available data and the ill-defined nature of the materials that were used in feeding studies.

PCDDs, PCDFs and coplanar PCBs were evaluated at the present meeting in response to a request by the Codex Committee on Food Additives and Contaminants at its Thirty-second Session (4) for the Expert Committee to evaluate the risks associated with their presence in food.

The Committee evaluated the PCDDs, PCDFs and coplanar PCBs for which toxic equivalency factors (TEFs) for mammals have been derived by WHO. Table 8 lists the compounds that were considered

Table 8

Compounds considered and their toxic equivalency factor assigned by WHO

Compound	Abbreviation	Toxic equivalency factor
Polychlorinated dibenzodioxins		
2,3,7,8-Tetrachlorodibenzodioxin	TCDD	1
1,2,3,7,8-Pentachlorodibenzodioxin	1,2,3,7,8-PeCDD	1
1,2,3,4,7,8-Hexachlorodibenzodioxin	1,2,3,4,7,8-HxCDD	0.1
1,2,3,6,7,8-Hexachlorodibenzodioxin	1,2,3,6,7,8-HxCDD	0.1
1,2,3,6,7,9-Hexachlorodibenzodioxin	1,2,3,6,7,9-HxCDD	0.1
1,2,3,4,6,7,8-Heptachlorodibenzodioxin	1,2,3,4,6,7,8-HpCDD	0.01
Octachlorodibenzodioxin	OCDD	0.0001
Polychlorinated dibenzofurans		
2,3,7,8-Tetrachlorodibenzofuran	2,3,7,8-TCDF	0.1
1,2,3,7,8-Pentachlorodibenzofuran	1,2,3,7,8-PeCDF	0.05
2,3,4,7,8-Pentachlorodibenzofuran	2,3,4,7,8-PeCDF	0.5
1,2,3,4,7,8-Hexachlorodibenzofuran	1,2,3,4,7,8-HxCDF	0.1
1,2,3,6,7,8-Hexachlorodibenzofuran	1,2,3,6,7,8-HxCDF	0.1
1,2,3,7,8,9-Hexachlorodibenzofuran	1,2,3,7,8,9-HxCDF	0.1
2,3,4,6,7,8-Hexachlorodibenzofuran	2,3,4,6,7,8-HxCDF	0.1
1,2,3,4,6,7,8-Heptachlorodibenzofuran	1,2,3,4,6,7,8-HpCDF	0.01
1,2,3,4,7,8,9-Heptachlorodibenzofuran	1,2,3,4,7,8,9-HpCDF	0.01
Octachlorodibenzofuran	OCDF	0.0001
“Non-ortho” polychlorinated biphenyls		
3,3',4,4'-Tetrachlorobiphenyl	3,3',4,4'-TCB	0.0001
3,4,4',5'-Tetrachlorobiphenyl	3,4,4',5'-TCB	0.0001
3,3',4,4',5-Pentachlorobiphenyl	3,3',4,4',5-PeCB	0.1
3,3',4,4',5,5'-Hexachlorobiphenyl	3,3',4,4',5,5'-HxCB	0.01
“Mono-ortho” polychlorinated biphenyls		
2,3,3',4,4'-Pentachlorobiphenyl	2,3,3',4,4'-PeCB	0.0001
2,3,4,4',5-Pentachlorobiphenyl	2,3,4,4',5-PeCB	0.0005
2,3',4,4',5-Pentachlorobiphenyl	2,3',4,4',5-PeCB	0.0001
2,3',4,4',5'-Pentachlorobiphenyl	2,3',4,4',5'-PeCB	0.0001
2,3,3',4,4',5-Hexachlorobiphenyl	2,3,3',4,4',5-HxCB	0.0005
2,3,3',4,4',5'-Hexachlorobiphenyl	2,3,3',4,4',5'-HxCB	0.0005
2,3',4,4',5,5'-Hexachlorobiphenyl	2,3',4,4',5,5'-HxCB	0.00001
2,3,3',4,4',5,5'-Heptachlorobiphenyl	2,3,3',4,4',5,5'-HpCB	0.00001

and their assigned TEF values. In the TEF approach, the toxicity of all chemicals in the series is related to that of 2,3,7,8-tetrachlorodibenzodioxin (TCDD), one of the most potent of the dioxins and that for which most toxicological and epidemiological information is available. Use of the TEF approach is based on the assumption that PCDDs, PCDFs and coplanar PCBs have a common mechanism of action, which involves binding to the aryl hydrocarbon (Ah) receptor, an intracellular receptor protein. This binding is considered to be the necessary, but not sufficient, first step in the expression of the toxicity of these compounds. Many uncertainties exist in applying the TEF approach to the assessment of human risk, but it is the most feasible approach currently available.

PCDDs, PCDFs and coplanar PCBs were considered by a WHO consultation held in 1998 (5), which established a tolerable daily intake (TDI) of 1–4pg/kg of body weight, applicable to the toxic equivalents of these compounds. The TDI was based on a number of studies of developmental toxicity and immunological toxicity. At its present meeting, the Committee used the assessment of the consultation as the starting point for its evaluation, taking into account the following additional data:

- the results of a study on the toxicokinetics of TCDD after single and repeated dosing;
- two new studies of developmental toxicity;
- new information on a study in rhesus monkeys that had been evaluated by the Committee at its fifty-fifth meeting (Annex 1, reference 149).

5.2.2 **Toxicokinetics**

5.2.2.1 *Absorption and biotransformation*

Coplanar compounds in dietary fat pass easily from the gut into the blood. Indeed, experiments in humans and laboratory animals given an oral dose of TCDD showed 50–90% absorption. This figure is comparable with the near-complete absorption of PCDDs, PCDFs and PCBs by nursing infants from their mothers' milk.

After absorption from the gut, TCDD enters the lymph in the form of chylomicrons and is cleared from the blood within 1 h, to appear mainly (74–81% of an administered dose) in the liver and adipose tissue. After clearance from the blood, coplanar compounds remain mainly in serum lipoproteins (very low density, low density and high density), and some are bound to serum proteins.

The Committee used the results of a study in which [³H]TCDD was given to pregnant Long-Evans rats by gavage as a single dose of 50,

200, 800 or 1000 ng/kg of body weight on day 15 of gestation, and the concentration of the radiolabel measured in tissues 1 day after treatment. The average maternal body burdens (with the percentage of the dose in the four treatment groups) were 31 (60%), 97 (48%), 520 (65%) and 580 (59%) ng/kg of body weight, respectively. On the basis of this study, the Committee used a value of 60% for the percentage of TCDD retained in pregnant rats 1 day after administration of a single dose by gavage on day 15 of gestation.

The distribution of PCDDs and PCDFs between the blood and organs is governed by lipid partitioning and binding to plasma proteins. The concentrations of PCDDs and PCDFs in blood and adipose tissue are closely correlated. TCDD is distributed between blood and adipose tissue by lipid partitioning, whereas the distribution of hexachlorodibenzodioxins (HxCDDs), hexachlorodibenzofurans (HxCDFs), octachlorodibenzodioxins (OCDDs) and octachlorodibenzofurans (OCDFs) is also governed by binding to plasma proteins.

Binding to plasma proteins plays an important role in the uptake of coplanar compounds from the blood in the liver, even for lower chlorinated congeners. When rodents are exposed to increasing doses of TCDD, it is preferentially sequestered in the liver. After entering liver cells, TCDD either dissolves in the lipid fraction or binds to the Ah receptor or to cytochrome P450 (CYP) proteins, probably microsomal CYP 1A2. As the amounts of CYP 1A and CYP 1B proteins in cells are regulated by formation of the TCDD–Ah receptor complex, exposure to increasing amounts of TCDD results in increased formation of this complex, which leads to increased production of CYP 1A and CYP 1B mRNA and proteins (enzyme induction), and accumulation of TCDD by increased binding to the induced CYP proteins. Similar sequestration has been observed with higher chlorinated PCDDs and PCDFs and with coplanar PCBs.

The hepatic sequestration of coplanar compounds markedly affects their distribution in the body. For example, whereas the liver usually contributes 10% and the adipose tissue 60% of the body burden of TCDD in mice, these fractions may increase to 67% in liver and decrease to 23% in adipose tissue in mice in which hepatic CYP proteins have been fully induced. Similar results were found in rats, clearly indicating the non-linear character of the kinetics of TCDD at concentrations that induce hepatic CYP proteins.

As in rodents, preferential sequestration of PCDDs and PCDFs in the liver rather than in adipose tissue has been observed in humans exposed to background concentrations of these compounds. Although Ah receptor-dependent CYP induction has been observed in

human liver cells in vitro after exposure to TCDD, it occurred at concentrations that were several orders of magnitude higher than those observed in human blood. It is therefore likely that the sequestration is due to binding to constitutive CYP proteins.

5.2.2.2 Metabolism and excretion

In laboratory animals, PCDDs and PCDFs are excreted almost exclusively in the bile, excretion in the urine being a minor route. Whereas the parent compound is found primarily in the organs of rodents, only metabolites of PCDDs and PCDFs occur in bile, indicating hepatic metabolism, including hydroxylation and conjugation, of these compounds. Similar reactions were found in vitro when recombinant human CYP 1A1 was incubated with TCDD. Faecal excretion of unmetabolized PCDDs and PCDFs is also an important route of elimination in humans.

In rodents, the half-life of TCDD ranges from 8–24 days in mice to 16–28 days in rats. Humans eliminate PCDDs and PCDFs more slowly, the estimated mean half-life of TCDD ranging from 5.5 to 11 years. The half-lives of other PCDD congeners and of PCDFs and coplanar PCBs vary widely. These differences in the half-lives of different congeners are reflected in their TEFs (see Table 8).

5.2.2.3 Relationship between human intake and doses used in studies in laboratory animals

The biochemical and toxicological effects of PCDDs, PCDFs and coplanar PCBs are directly related to their concentrations in tissues, and not to the daily dose. The most appropriate measure of dose would therefore be the concentration at the target tissue; however, this is seldom known. The body burden, which is strongly correlated with the concentrations in tissue and serum, integrates the differences in half-lives between species. Thus, rodents require appreciably higher daily doses (100–200-fold) to achieve a body burden at steady state that is equivalent to that recorded in humans exposed to background concentrations. Toxicokinetically, estimates of body burden are therefore more appropriate measures of dose for interspecies comparisons than is the daily dose.

The long half-lives of PCDDs, PCDFs and coplanar PCBs in humans have several implications for the period of intake that is relevant to the assessment. First, the concentration of toxic equivalents in the body (or the internal toxic equivalents to which a target organ is exposed) will increase over time as more of the compounds are ingested. Second, after cessation of exposure, the body's concentration of stored toxic equivalents (and the exposure of internal organs) will

decline slowly, only half of the accumulated toxic equivalents disappearing over about 7 years, resulting in a pseudo-steady state only after decades. Third, because of this long-term storage in the body and the consequent daily exposure to the body's stored toxic equivalents, intake on a particular day will have a small or even negligible effect on the overall body burden. For example, in the unlikely event of food contamination that leads to an intake 100 times the amount present in a typical meal, the body burden of the adult eating that meal would increase by <3%. The rest of the body burden would be made up of the PCDDs, PCDFs and coplanar PCBs consumed in many thousands of meals over the previous decade or more.

Therefore, the Committee concluded that the appropriate period for evaluating the mean intake of these compounds is 1 month.

In order to transform an animal body burden into an equivalent human monthly intake (EHMI) that on a long-term basis would result in a similar body burden (at steady state), simple, classical toxicokinetic calculations can be used. The elimination of low doses of PCDDs was considered to follow first-order kinetics and to be independent of the body burden or dose. The Committee calculated the total body burden at steady-state using the following equation:

$$\text{Body burden at steady state (ng/kg of body weight)} = \frac{f \times \text{intake (ng/kg of body weight per day)} \times \text{half-life (days)}}{\ln(2)}$$

where *f* is the fraction of dose absorbed from food (assumed to be 50% in humans) and the estimated half-life of TCDD is 2774 days (7.6 years). For compounds that follow first-order kinetics, 4–5 half-lives will be required to approach steady state. For TCDD, this would be equivalent to more than 30 years.

This model is based on the assumption that PCDDs are distributed in only one compartment: the whole body. Although most of the body burden of PCDD is distributed in the lipid stores, at higher doses the liver also sequesters these compounds in both humans and animals. Predictions of body burden after intake of high doses that are based on lipid concentrations may therefore be underestimates of the total body burden (and the intake leading to that body burden), because of hepatic sequestration. Use of physiologically based pharmacokinetic models may be more appropriate under these circumstances. In order to transform the body burdens resulting from intake of the low concentrations to which the general population is exposed and from the low doses used in the pivotal toxicological studies into estimated

human daily intake, the Committee considered use of a less complicated, classical pharmacokinetic model to be appropriate.

5.2.2.4 Determinants of dose received by fetuses in studies of developmental toxicity

The time of dosing in several of the studies considered by the Committee, day 15 of gestation, marks the onset of the sensitive phase of sexual differentiation in rats and represents a critical time of fetal exposure. The determinant of the reproductive effects is the fetal concentration on days 15–16 of gestation, which in turn is determined by the maternal serum concentration. The latter concentration differs with a bolus dose (as in these studies) and with repeated doses providing the same total intake. As the serum concentration of TCDD after a bolus dose rises before distribution to the tissue compartments, the serum concentration is likely to be higher than that after long-term intake of a lower concentration.

The difference in the fetal body burden after a single bolus dose and after repeated administration of low doses resulting in a similar maternal body burden was addressed in a study in Long-Evans rats treated on day 16 of gestation (6, 7). The rats were given [³H]TCDD at 1, 10 or 30 ng/kg of body weight per day by gavage in corn oil, on 5 days per week for 13 weeks. They were then mated, and dosing was continued daily throughout gestation. The regimen produced a steady-state concentration of TCDD in the dams. The average maternal and fetal body burdens on day 16 of gestation after this treatment and after administration of a single dose of TCDD by gavage on day 15 of gestation are shown in Table 9.

As expected, a single dose on day 15 of gestation by gavage resulted in considerably higher fetal concentrations on day 16 than short-term administration of low daily doses leading to maternal steady-state body burdens of similar magnitude.

Using the data in Table 9, the Committee conducted least-squares linear fits of dose versus maternal and fetal body burdens. Since radiolabelled TCDD was used in both studies, a zero intercept was assumed for the fitted line. None of these fits showed what appeared to be any significant deviation from linearity. These data indicate that the ratio of fetal:maternal body burden resulting from a bolus dose would be 1.7 times that from multiple doses providing the same total dose. Kinetic data indicate that a linear dose–response relationship would be expected at the doses used in these studies. The fetal and maternal body burdens in both data sets were also fitted to power equations, which provided a better fit of the data obtained at the low end of the range of single doses. The factor used to convert maternal

Table 9

Average maternal and fetal body burdens after a single dose and after administration of repeated doses of TCDD to pregnant Long-Evans rats

Dose (ng/kg of body weight per day)	Body burden on day 16 of gestation (ng/kg of body weight per day)	
	Maternal	Fetal
Single dose		
50	30	5.3
200	97	13
800	520	39
1000	520	56
Repeated doses^a		
0.71	20	1.4
7.1	120	7.5
21	300	15

Source: references 6 and 7; used by permission.

^a Daily dose, adjusted for continuous administration from 5 to 7 days per week.

body burden after single doses to a corresponding steady-state body burden with the power equations was 2.6.

5.2.3 *Toxicological studies*

5.2.3.1 *Acute toxicity studies*

In laboratory animals, the acute toxicity of TCDD and related PCDDs and PCDFs substituted in at least the C-2, C-3, C-7 and C-8 positions varies widely between and among species. For example, the median lethal dose in guinea-pigs treated orally was 0.6 µg/kg of body weight, while that in hamsters was >5000 µg/kg of body weight. Explanations for this variation include differences in Ah receptor functionality (size, transformation and binding of the PCDD response element), toxicokinetics (metabolic capacity and tissue distribution) and body fat content. While data on acute toxicity were available for various commercial PCB mixtures (median lethal doses usually >100 mg/kg of body weight), the data on individual coplanar PCB congeners in mammals were limited.

One of the more common symptoms associated with lethality induced by PCDDs is a generalized delayed wasting syndrome characterized by inhibition of gluconeogenesis, reduced feed intake and loss of body weight. Other toxic effects observed after a single exposure to PCDDs include haemorrhages in a number of organs, thymic atrophy, reduced bone-marrow cellularity and loss of body fat and lean muscle mass, although some differences in the frequency of these effects was seen among species.

5.2.3.2 Carcinogenicity studies

TCDD and other PCDDs induced tumours at multiple sites in laboratory animal species of each sex. In a series of assays *in vivo* and *in vitro*, TCDD promoted the growth of transformed cells (e.g. rat tracheal epithelium cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine), consistent with observations of cancer promotion in whole animals *in vivo*. In a long-term study of carcinogenicity with TCDD in rats, the LOEL for hepatic adenomas in females was 10 ng/kg of body weight per day, and the NOEL was 1 ng/kg of body weight per day. Several studies have shown that TCDD promotes tumours in laboratory animals, in particular liver tumours. Several other PCDDs, PCDFs and non-*ortho*- and mono-*ortho*-PCBs also promoted liver tumours. In a long-term study in rats in which the incidence of liver tumours was increased over that in controls, the LOEL of 10 ng/kg of body weight per day corresponded to a steady-state body burden of 290 ng/kg of body weight. In order for humans to attain a similar steady-state body burden, they would have to have a daily intake of 150 pg/kg of body weight (see the equation on page 126).

5.2.3.3 Genotoxicity studies

The results of several short-term assays for genotoxicity with TCDD, covering various end-points, were negative. Furthermore, TCDD did not bind covalently to DNA from the liver of mice. The Committee concluded that TCDD does not initiate carcinogenesis.

5.2.3.4 Developmental toxicity studies

A number of biochemical changes, including enzyme induction, altered expression of growth factors and enhanced oxidative stress, have been noted in laboratory animals with body burdens of TCDD within a lower range of 3–10 ng/kg of body weight. The Committee considered these biochemical effects to be early markers of exposure to PCDDs, PCDFs and coplanar PCBs, or events induced by these compounds in animals and in humans that may or may not result in adverse effects at higher body burdens.

The Committee reviewed the relevant studies (8–14) considered by the WHO consultation held in 1998 (4), as well as three recent studies (15–17). The Committee noted that the most sensitive adverse effects reported were on development in the male offspring of rats and immunological deficits in rats after prenatal exposure to TCDD (see Table 10).

The WHO consultation identified a study in which endometriosis was found after long-term administration of TCDD to rhesus monkeys. The Committee stressed that the findings in this study should be

Table 10

Summaries of studies in which the lowest NOELs and LOELs were identified for the most sensitive adverse effects of TCDD on developmental end-points in rats^a

Dosing regimen	Strain	End-point	NOEL body burden (ng/kg of body weight)	LOEL body burden (ng/kg of body weight)	Reference no.
Single bolus by gavage on day 14 of gestation	Fischer 344	Immune suppression in offspring		50	8, 15
Single bolus by gavage on day 15 of gestation	Holtzman	Reduced ventral prostate weight; decreased anogenital distance in male offspring	13	51	17
Single bolus by gavage on day 15 of gestation	Holtzman	Decreased sperm count in offspring		28	13
Single bolus by gavage on day 15 of gestation	Long-Evans	Accelerated eye opening and decreased sperm count in offspring		28	9
Loading and maintenance doses by subcutaneous injection	Wistar	Decreased sperm production and altered sexual behaviour in male offspring		25	16

^a Body burdens estimated from a linear fit to the data in Table 9.

interpreted with caution, as the daily intake was not adequately reported. In addition, analyses conducted 13 years after the end of exposure showed high concentrations of coplanar PCBs in the blood of the monkeys with endometriosis, possibly from an unknown source (14). The Committee also noted that the LOELs in some of the pivotal studies in rats (Table 10) would result in EHMI that were similar to or lower than that derived from the LOEL for endometriosis in monkeys.

In a recent study (17), pregnant Holtzman rats were given a single oral dose of TCDD at 0–800 ng/kg of body weight on day 15 of gestation, and the male offspring were examined on days 49 and 120 after birth. No changes were seen in testicular or epididymal weight nor in daily sperm production or sperm reserve at any dose. However, the weight of the urogenital complex, including the ventral prostate, was significantly reduced at doses of 200 and 800 ng/kg of body weight in rats killed on day 120. Moreover, the anogenital distance of male rats receiving doses ≥ 50 ng/kg of body weight and killed on day 20 was significantly decreased. The Committee noted that administration of TCDD at any dose resulted in a dose-dependent increase in 5 α -reductase type 2 mRNA and a decrease in androgen receptor mRNA in the ventral prostate of rats killed at day 49 but not in those killed at day 120, with no adverse sequelae at the lowest dose of 12.5 ng/kg of body weight. On the basis of 60% absorption and an assumption of a linear relationship for the data in Table 9, the equivalent maternal body burden after multiple doses at this NOEL would be 13 ng/kg of body weight. Fitting the data in Table 9 into the power equation, the Committee estimated the body burden NOEL to be 19 ng/kg of body weight. The LOEL of 50 ng/kg of body weight per day corresponds to an equivalent body burden of 51 ng/kg of body weight with the linear model and 76 ng/kg of body weight with the power model.

The lowest LOEL reported for the reproductive system of male offspring was found in an experiment with Wistar rats (16). In this study, the dams were treated subcutaneously before mating and throughout mating, pregnancy and lactation. They received an initial loading dose of [^{14} C]TCDD at 25, 60 or 300 ng/kg of body weight 2 weeks before mating, and then a weekly maintenance dose of TCDD at 5, 12 or 60 ng/kg of body weight. The size of the maintenance doses was determined on the basis of a reported elimination half-life for TCDD of 3 weeks in adult rats. The effects on male reproductive end-points were studied on days 70 and 170 after birth. The number of sperm per cauda epididymis at puberty and in adulthood was lower in the offspring of all treated dams than in those of controls. Daily sperm production was permanently lower in offspring of treated dams than

in those of controls, as was the sperm transit rate, thus increasing the time required by the sperm to pass through the cauda epididymis. Moreover, the offspring of the treated groups showed increased numbers of abnormal sperm when investigated in adulthood. The latency periods to mounting and intromission were significantly greater in offspring of dams at the lowest and highest doses, but not of those at the intermediate dose, than in offspring of controls. The Committee noted the lack of clear dose–response relationships for most of these effects in the treated groups. In the male offspring of dams at the highest dose, the concentration of serum testosterone was decreased in adulthood, and permanent changes found in the testicular tubuli included pyknotic nuclei and the presence of cell debris in the lumen. The fertility of the male offspring was not affected in any of the treated groups.

In computing the long-term dose required to produce the fetal concentration found in the group given the initial loading dose of 25 ng/kg of body weight, the Committee noted that the dose would have been reduced to 20 ng/kg of body weight before the maintenance dose of 5 ng/kg of body weight given on day 14. On the basis of the linear fit to the data in Table 9, the fetal body burden resulting from the maternal body burden of 20 ng/kg of body weight would be 1.04 ng/kg of body weight. The maintenance dose of 5 ng/kg of body weight administered on day 14 of gestation would make an additional contribution to the fetal body burden of 0.27 ng/kg of body weight, resulting in a total fetal body burden of 1.31 ng/kg of body weight. On the basis of a linear fit to the data in Table 9, a maternal body burden of TCDD of 25 ng/kg of body weight at steady state would be required to produce this fetal body burden.

The studies summarized in Table 10 provide evidence that adverse effects on the reproductive system are induced in male offspring of pregnant rats given TCDD. The studies show reductions in daily sperm production, in the number of sperm in the cauda epididymides and in epididymal weight as well as accelerated eye opening, a reduction in anogenital distance and feminized sexual behaviour in male offspring associated with maternal steady-state body burdens of TCDD of ≥ 25 ng/kg of body weight. Reductions in the weights of the testes and the size of the sex accessory glands, such as the ventral prostate, in male offspring, development of external malformations of the genitalia in female offspring and reduced fertility in males and females required higher maternal body burdens.

The Committee noted that the most sensitive end-points differed between studies, perhaps reflecting strain differences in sensitivity and even minor differences in the experimental conditions, e.g. the

diet. The Committee also noted that, in one study, administration of a single dose of TCDD at 12.5 ng/kg of body weight to dams by gavage decreased the amount of androgen receptor mRNA in the ventral prostate of offspring at puberty on day 49 after birth, indicating reduced androgenic responsiveness. However, none of the other above-mentioned adverse effects were seen in male offspring at this dose, which corresponds to an estimated maternal steady-state body burden of TCDD of approximately 19 ng/kg of body weight (Table 10). The Committee considered the effect on androgenic responsiveness to be an early marker of exposure to TCDD, like enzyme induction, altered expression of growth factors and enhanced oxidative stress, or an event that may or may not result in adverse effects in animals at higher body burdens.

5.2.4 **Observations in humans**

5.2.4.1 Effects other than cancer

In two episodes of food poisoning in China (Province of Taiwan) and Japan, in which infants were exposed in utero to heat-degraded PCBs, a variety of adverse physical developmental abnormalities was observed, including decreased penis length and alterations of spermatozoa; neurodevelopmental abnormalities were also seen. The affected children in Taiwan, China, were born to mothers with estimated body burdens of toxic equivalents of PCBs of 2–3 µg/kg of body weight.

Environmental or background exposure of infants in Germany, the Netherlands and the USA was evaluated in several studies; for example, the mean concentration of toxic equivalents in human milk was 60 pg/g of lipid (range 25–155 pg/g) in a study in Groningen and Rotterdam, the Netherlands. Low birth weight and detriments in neurological development and alterations in thyroid hormones, the distribution of lymphocyte subpopulations and the frequency of infections and respiratory symptoms were observed. The observed neurodevelopmental deficits were subtle and the prevalence within the normal range; their potential consequences for future intellectual function are unknown. The associations observed were considered to be due to prenatal exposure rather than to postnatal intake (from milk). In one study of breastfed and bottle-fed infants, the intake of PCDDs and PCBs was inversely related to performance in neurobehavioural tests, breastfed infants having better scores than bottle-fed infants. These studies of low exposure related primarily to PCBs, and fewer data were available on the effects of PCDDs and PCDFs.

In adults, most of the effects other than cancer observed after exposure to PCDDs, PCDFs and coplanar PCBs, such as chloracne,

appeared only at doses several orders of magnitude greater than those generally received from background contamination of foods. In Seveso, Italy, more female children than expected were born to fathers who had serum TCDD concentrations $>80\text{pg/g}$ of lipid ($16\text{--}20\text{ng/kg}$ of body weight) at the time of conception.

5.2.4.2 Carcinogenicity

A working group convened by the International Agency for Research on Cancer (IARC) classified TCDD as a human carcinogen (Group 1). Other PCDDs and PCDFs were considered not to be classifiable as to their carcinogenicity to humans (Group 3).

The most informative studies for evaluating the carcinogenicity of TCDD are four cohort studies of herbicide producers (two in Germany and one each in the Netherlands and the USA) and one cohort study of residents of a contaminated area in Seveso, Italy. A multi-country cohort study from IARC included three of these four cohorts, other industrial cohorts, many of which had not been reported in separate publications, and a cohort of professional herbicide applicators.

In most of the epidemiological studies considered, exposure had been primarily to TCDD, with some exposure to mixtures of other PCDDs, as contaminants of phenoxy herbicides and chlorophenols. The studies involved persons with the highest recorded exposure to TCDD, the estimated geometric mean blood lipid concentrations after the last exposure ranging from 1100 to 2300pg/g of lipid in the industrial cohorts; lower average concentrations were found in the population exposed in Seveso.

Low excess risks of the order of 40% were found for all neoplasms combined in all the studies of industrial cohorts in which the exposure assessment was adequate. The risks for cancers at specific sites were increased in some of the studies, but the results were not consistent between studies, and no single cancer site seemed to predominate. The results of tests for trends for increasing excess risks for all neoplasms with increasing intensity of exposure were statistically significant. Increasing risks for all neoplasms with time since first exposure were observed in those studies in which latency was evaluated. The follow-up of the Seveso cohort has so far been shorter than that of the industrial cohorts; however, the rate of death from all cancers has not been found to differ significantly from that expected in the general population. Excess risks were seen for cancers at some specific sites among persons in the most heavily contaminated zones at the time of the accident, but there were few cases.

In these well-conducted cohort studies, the intensity of exposure could be ascertained with precision because of the long biological half-life of TCDD in human tissues, and the relative risks increased significantly with increasing exposure. Although the excess cancer risk at the highest exposure was statistically significant, these results must be evaluated with caution, as the overall risks are not high and the strongest evidence is for industrial populations whose exposure was two to three orders of magnitude greater than that of the general population, and who also had heavy exposure to other chemicals; furthermore, lifestyle factors such as smoking were not evaluated. There are few precedents of carcinogens that increase the risk for cancer at all sites combined, with no excess risk for any specific tumour predominating.

A “benchmark dose” was calculated from the effective dose estimated to result in a 1% increase in cancer mortality (ED_{01}), on the basis of a meta-analysis of data for three industrial cohorts with well-documented exposure and comparison with the doses required for effects other than cancer. A statistically significant linear trend in risk with intensity of exposure was observed, which persisted even after exclusion of the groups with the greatest exposure. Within the range of reasonable assumptions, the ED_{01} differed quite widely and depended strongly on the assumptions made. Furthermore, a number of uncertainties would influence the predicted ED_{01} , including the exact exposure of the occupational cohorts and, to a lesser extent, the potential confounding effects of factors not considered in the studies.

5.2.5 ***Sampling and analytical methods***

As no specific guidelines have been drawn up for sampling foods to be analysed for their PCDD, PCDF and coplanar PCB content, the basic guidelines for sampling for organic contaminants or pesticides should be used. The objective is to obtain a representative, homogeneous laboratory sample without introducing secondary contamination. Although PCDDs, PCDFs and coplanar PCBs are chemically stable, the samples should be stored and transported in such a way that they do not deteriorate. PCDDs, PCDFs and coplanar PCBs are usually found as complex mixtures of varying composition in different matrices. Their identification and quantification require a highly sophisticated method of analysis in order to separate the toxic congeners listed in Table 8 from the more prevalent, less toxic congeners. Usually, PCDDs, PCDFs and coplanar PCBs are determined by capillary gas chromatography with mass spectrometry.

No official method exists for the determination of these compounds in food. Reliable results have been obtained in the absence of official

methods when the method used has been shown to be suitable and to fulfil analytical quality criteria developed in other fields of residue analyses. The methods used to determine PCDDs and PCDFs in food must provide sufficient information to allow calculation of the results as toxic equivalents, at concentrations of 0.1–1 pg/g of fat in milk, meat and eggs, 10 pg/g of fat in fish or ≥ 100 pg/g of fat in cases of heavier contamination, and 0.1–0.5 pg/g of dry matter in food of vegetable origin. The patterns of congeners can vary between regions and foods.

When the method used is of insufficient sensitivity, the concentrations of PCDDs, PCDFs and coplanar PCBs in many foods may be near or below the limit of quantification. The method used to derive the concentrations of undetected congeners (the imputation method) can therefore have a variable effect on the summary toxic equivalent value for a food sample. In the most commonly used imputation methods, the contribution of each undetected congener to the toxic equivalent is considered to be either 0 (“lower-bound concentrations”), the limit of detection or limit of quantification (“upper-bound concentrations”) or half the limit of detection or limit of determination. In methods with insufficient sensitivity, the lower- and upper-bound concentrations can differ by a factor of 10–100 or even more. If the sensitivity is appropriate, the differences between lower- and upper-bound concentrations are negligible. Therefore, low estimates of PCDDs, PCDFs and coplanar PCBs in a sample may represent truly low concentrations or be the result of use of zero as the value for undetected congeners in a food sample. Conversely, high estimates may be the result either of contamination or of use of the upper-bound concept with insufficient sensitivity.

Application of upper-bound or lower-bound concentrations leads to over- and underestimates of intake, respectively. Therefore, the Committee recommended that laboratories report their results as lower-bound, upper-bound and half-detection limits, in addition to values for individual congeners, thus providing all the necessary information for interpreting the results for specific requirements. Experts who are summarizing results based on toxic equivalents should indicate the way in which the toxic equivalents were calculated.

For analysis of food samples with normal background contamination by PCDDs, PCDFs or PCBs, gas chromatography with high-resolution mass spectrometry has been validated in collaborative studies and has been shown to provide the required sensitivity and specificity. Bioanalytical assays have been developed for rapid screening of sediments, soil, fly ash and various foods, but only the chemical-

activated luciferase gene expression (CALUX) assay has been used for food; validation of this assay has begun. While gas chromatography with mass spectrometry is the most powerful method for identifying and quantifying congeners and for recognizing congener-specific patterns, it does not allow direct measurement in a matrix of all congeners present that act through the Ah receptor pathway. The CALUX assay provides an indication of the toxic equivalents present in a certain matrix, including interactive (synergistic or antagonistic) effects; however, it cannot provide information on the pattern of congeners.

The Committee recognized that the available analytical data on PCDDs, PCDFs and coplanar PCBs are limited by the lack of generally accepted criteria for intra- and inter-laboratory validation. Mutual acceptance of analytical methods would be facilitated by international collaborative studies and proficiency testing programmes. For reliable analysis of concentrations in the range of normal background contamination, control laboratories must use sufficiently sensitive methods. General statistical parameters that have been established in other fields of residue analysis could be used. The requirements for acceptable analytical methods clearly need to be harmonized, so that data are comparable and can be used for risk management purposes.

5.2.6 *Levels and patterns of contamination of food commodities*

Data were submitted by Belgium, Canada, Japan, New Zealand, Poland and the USA and by the European Commission in a report containing data on Belgium, Denmark, Finland, France, Germany, Italy, the Netherlands, Norway, Sweden and the United Kingdom. In all countries in which a substantial number of samples had been analysed, the concentrations of PCDDs, PCDFs and coplanar PCBs in food were found to have decreased up to the late 1990s, but the decrease had slowed or was even partly reversed in some food categories in several countries owing to contamination of animal feed. For the present assessment of intake at the international level, only data collected after 1995 were considered.

As the Committee did not have access to the original analytical results, it was not possible to ascertain whether the results had been obtained by the lower- or upper-bound approach, and the concentrations used in the assessment were expressed as sums of congeners.

Insufficient individual data were available from most countries to allow construction of a full curve of the distribution of concentrations. Most data were submitted in an aggregated format. As recommended by a FAO/WHO workshop on assessing exposure to contaminants

Table 11

Weighted mean and derived median concentrations of PCDDs, PCDFs and coplanar PCBs in six food groups, expressed as toxic equivalents (pg/g whole food)

Region or country	Food category	PCDDs/PCDFs		Coplanar PCBs	
		Weighted mean	Derived median	Weighted mean	Derived median
North America	Dairy	0.10	0.07	0.02 ^a	0.01 ^a
	Eggs	0.17	0.14	0.04 ^a	0.02 ^a
	Fish	0.56	0.28	0.13 ^a	0.08 ^a
	Meat	0.13	0.10	0.14 ^a	0.05 ^a
Western Europe	Dairy	0.07	0.04	0.08	0.07
	Eggs	0.16	0.15	0.07	0.06
	Fish	0.47	0.31	2.55	0.90
	Meat	0.08	0.06	0.41	0.08
	Vegetable products	0.04	0.03	0.04	LOD
Japan	Dairy	0.06	0.04	0.04	0.02
	Eggs	0.07	0.03	0.06	0.04
	Fish	0.37	0.11	0.69	0.19
	Meat	0.09	0.01	0.04	0.009
	Vegetable products	0.003	0.002	0.02	0.003
New Zealand	Dairy	0.02	0.02	0.01	0.008
	Fish	0.06	0.05	0.09	0.07
	Meat	0.01	0.01	0.02	0.01
	Vegetable products	0.008	0.008	<LOD	<LOD
All	Fats and oils	0.21	0.10	0.07 ^a	0.02

LOD: limit of detection.

^a Data on PCBs frequently did not include mono-*ortho* PCBs.

(18), aggregated data were weighted as a function of the number of initial samples and then used to obtain a weighted mean concentration of PCDDs, PCDFs and PCBs in six major food groups: meat and meat products, eggs, fish and fish products, milk and milk products, vegetables and vegetable products, and fats and oils. National data were aggregated by region or country (North America, Western Europe, Japan and New Zealand) and are summarized in Table 11. Insufficient data were available for the rest of the world to permit a realistic estimate of the distribution of contaminants. The Committee recognized that there are significant differences within the food categories in Table 11, and that the data used in this analysis may not reflect the true mean for a food category. For example, the mean concentrations of PCDDs, PCDFs and coplanar PCBs and the rate of consumption vary considerably in different fish species, and it was not possible to determine if the mean represents the fish species most

commonly consumed. However, the data received were not sufficient to allow an analysis that might account for such variation.

In a second step, a log-normal distribution of contaminants in foods was assumed, and a model of distribution was constructed from the weighted mean and a geometric standard deviation of 3 derived from the concentrations in six broad food groups. On the basis of these derived distributions, the percentiles of consumption were determined. The derived median values (50th percentiles) are presented in Table 11.

5.2.7 *Estimated dietary intake*

Because of the long half-lives of PCDDs, PCDFs and coplanar PCBs, their hazard to health can be estimated only after consideration of intake over a period of months. Short-term variations in PCDD, PCDF and coplanar PCB concentrations in foods have much less effect on overall intake than might be the case for other food contaminants.

The distribution of long-term mean intake in various populations was calculated by the following procedure:

- The distributions of concentrations were constructed for various regions and food groups from the available data. The distributions were assumed to be log-normal.
- Data on food consumption from the GEMS/Food regional diets and national surveys were used to estimate mean consumption of six major food groups in each diet. A log-normal distribution was constructed from these data with a geometric standard deviation of 1.3 extrapolated from the results of the food consumption survey in the Netherlands in order to account for inter-individual variation in consumption. The average contributions of the six basic food groups to total food consumption were derived for each diet.
- The dietary intake of a particular population was assessed by combining the concentrations in foods and food consumption distributions for that population using a Monte Carlo approach. In each Monte Carlo trial, the dietary intake was estimated by multiplying random values for food consumption and concentrations in various food groups. The concentrations were weighted according to the contribution of the food group to total food consumption. The estimates of intake were combined to form a distribution of long-term mean dietary intake for each population studied. The distributions are characterized by a median and a 90th percentile intake. Calculations were performed for the sum of the toxic equivalents of PCDDs and PCDFs and for the sum of coplanar PCBs separately, because the data on occurrence of PCBs were obtained independently.

Table 12

Median and 90th percentile values of estimated long-term intake of PCDDs, PCDFs and coplanar PCBs,^a based on the GEMS/Food regional diets

Source of data on concentrations ^b	Source of data on food consumption	Intake of PCDDs and PCDFs		Intake of coplanar PCBs	
		Median	90th percentile	Median	90th percentile
North America	Europe	68	160	14	35
Western Europe	Europe	54	130	57	150
Japan	Far East	7	15	7	19
New Zealand	Europe	18	36	10	22

^a Expressed as toxic equivalents, pg/kg of body weight per month, assuming 60kg of body weight.

^b For North America, the data on concentrations in vegetables in Western Europe were used; for New Zealand, the data on concentrations in eggs in Japan were used.

Table 13

Median and 90th percentile values of estimated long-term intake of PCDDs, PCDFs and coplanar PCBs,^a based on national food consumption data

Source of data on concentrations ^b	Source of data on food consumption	Intake of PCDDs and PCDFs		Intake of coplanar PCBs	
		Median	90th percentile	Median	90th percentile
North America	USA	42	100	9	25
Western Europe	France	40	94	47	130
	Netherlands	33	81	30	82
	United Kingdom	39	91	41	110

^a Expressed as toxic equivalents, pg/kg of body weight per month, assuming 60kg of body weight.

^b For North America, the data on concentrations in vegetables in Western Europe were used.

The simulated intakes of PCDDs, PCDFs and coplanar PCBs in the GEMS/Food regional diets are presented in Table 12. These intakes are, however, likely to be overestimates, as the data on concentrations were derived from surveys (without random sampling) and from the GEMS/Food regional diets, which are based on data on food supply (apparent consumption) and which are known to overestimate food consumption by at least 15%.

More reliable estimates of intake (Table 13) were obtained by using national food consumption data rather than data on the food supply (apparent consumption) from the GEMS/Food regional diets. The simulated intakes presented in Table 13 are not strictly national estimates and are somewhat higher than the national estimates submitted by the European Commission.

The calculated contributions of various food categories to the intake of PCDDs, PCDFs and coplanar PCBs showed that the largest fraction (>70%) is from food of animal origin in both the GEMS/Food regional diets and the national diets.

Information was lacking on both the quality of data and geographical representativeness for some regions. More data are required on the occurrence of coplanar compounds in food products, particularly from geographical regions other than Europe, so that more representative estimates of intake can be made for all regions.

Breastfed infants have higher intakes of these compounds than bottle-fed infants or adults on a body-weight basis, although for only a small portion of their lives. Breast milk has beneficial effects, despite the risk of contamination. WHO has therefore repeatedly evaluated the health significance of contamination of breast milk with coplanar compounds. WHO recommends and supports breastfeeding but has concluded that continued and enhanced efforts should be directed towards identifying and controlling environmental sources of these substances.

5.2.8 **Evaluation**

In view of the long half-lives of PCDDs, PCDFs and coplanar PCBs, the Committee concluded that it would not be appropriate to establish an acute reference dose for these compounds.

The Committee concluded that a tolerable intake could be established for TCDD on the basis of the assumption that there is a threshold for all effects, including cancer. Carcinogenicity due to TCDD was not linked to mutagenicity or DNA binding, and it occurred at higher body burdens in animals than other toxic effects. The Committee concluded that the establishment of a tolerable intake based on effects other than cancer would also address any carcinogenic risk.

The studies listed in Table 10 were those considered by the Committee in choosing the lowest LOELs and NOELs for assessment of tolerable intake. The lowest LOEL and NOEL were provided by the studies of Faqi et al. (16) and Ohsako et al. (17), respectively. With the toxicokinetic conversions described in Table 9, these two studies indicate maternal body-burden LOELs and NOELs for effects on male rat offspring of 25ng/kg of body weight and 13ng/kg of body weight, respectively.

5.2.8.1 *Background body burdens in laboratory animals*

In the studies used to estimate body burden on the basis of the distribution of TCDD after multiple dosing, radiolabelled material

was used. Therefore, the known background concentrations of TCDD and other PCDDs and PCDFs in the tissues of laboratory rodents resulting from traces of these compounds in rat feed were ignored. The Committee identified two studies that could be used to predict the body burdens of rats resulting from the presence of coplanar compounds in laboratory feed. These studies were mutually consistent and predicted that “unexposed” laboratory rats had toxic equivalent body burdens of 3–12 ng/kg of body weight, depending on age. Thus, the maternal body burdens of TCDD seen in studies with radiolabelled material should be adjusted upwards by a minimum of 3 ng/kg of body weight to account for the background concentrations of unlabelled PCDDs and PCDFs. The maternal toxic equivalent body burden may still be underestimated, as 3 ng/kg of body weight was the minimum in the two studies, and in one of the studies coplanar PCBs were not included.

Addition of 3 ng/kg of body weight to the body burdens calculated from the linear model and the data in Table 9 resulted in estimated total toxic equivalent body burdens of 16 ng/kg of body weight for the NOEL and 28 ng/kg of body weight for the LOEL. These body burdens correspond to EHMI of 240 and 420 pg/kg of body weight, respectively. Fitting the data in Table 9 into the power-model equation gave EHMI of 330 pg/kg and 630 pg/kg of body weight, respectively.

5.2.8.2 Identification of safety factors

The safety factors considered in establishing acceptable levels of intake on the basis of the results of studies in laboratory animals usually include the following: a factor to convert a LOEL to a NOEL (if needed); a factor to extrapolate from animals to humans; and factors to account for inter-individual variations in susceptibility. Typically, factors of 10 have been used for extrapolation between species and for accounting for the human variation in susceptibility, and a factor of 3–10 for extrapolating from a LOEL to a NOEL.

As a NOEL was identified for effects in the male offspring of rats, no factor for conversion from a LOEL to a NOEL was needed for the EHMI derived from the study described above (17).

As concluded by the WHO consultation (5), use of body burdens to scale doses from studies in laboratory animals to equivalent human doses removes the need for safety factors to account for differences in toxicokinetics between animals and humans.

To account for inter-individual differences in toxicokinetics among humans, a safety factor should be applied. The Committee noted that limited data were available on the toxicokinetics of TCDD in humans and considered that the default factor of 3.2 was appropriate.

The Committee observed that humans may be less sensitive than rats to some effects. However, the conclusion is less certain for other effects and the possibility that the most sensitive humans might be as sensitive to the adverse effects of TCDD as rats were in the pivotal studies cannot be excluded. Therefore, the Committee concluded that no safety factor in either direction need be applied for differences in toxicodynamics among humans.

Use of a LOEL instead of a NOEL indicates the need for an additional safety factor. As the LOEL for the sensitive end-point was considered to be close to a NOEL and represented marginal effects, the Committee applied a factor of 3 to account for use of a LOEL instead of a NOEL. This resulted in an overall safety factor of 9.6 (3×3.2).

The Committee concluded that a total safety factor of 3.2 should be applied to the EHMI associated with the NOEL, and a total safety factor of 9.6 should be applied to the EHMI associated with the LOEL.

5.2.8.3 Tolerable intake

As stated in the discussion of toxicokinetics, the long half-lives of PCDDs, PCDFs and coplanar PCBs mean that each daily ingestion has a small or even a negligible effect on overall intake. In order to assess long- or short-term risks to health due to these substances, total or average intake should be assessed over months, and tolerable intake should be assessed over a period of at least 1 month. To encourage this view, the Committee decided to express the tolerable intake as a monthly value in the form of a provisional tolerable monthly intake¹ (PTMI).

As shown in Table 14, use of the linear model to extrapolate the maternal body burden at the NOEL, obtained with a single dose, to that expected at multiple doses gives a EHMI of 237 pg/kg of body weight, which would be expected to result in a body burden that is lower than that which had effects in animals. The PTMI derived by application of the safety factor of 3.2 to this EHMI is 74 pg/kg of body weight.

Similarly, as shown in Table 14, the PTMI derived by application of the safety factor of 9.6 to the EHMI derived from the study that

¹ By analogy with the provisional tolerable weekly intake (PTWI), the end-point used for safety evaluations by the Committee for food contaminants with cumulative properties. Its value represents the permissible human monthly exposure to those contaminants unavoidably associated with otherwise wholesome and nutritious foods.

Table 14

Summary of four calculations of PTMI

	Linear model		Power model	
	NOEL	LOEL	NOEL	LOEL
Administered dose (ng/kg of body weight)	12.5 ^a		12.5 ^a	
Maternal body burden (ng/kg of body weight)	7.6	25 ^b	7.6	25 ^b
Equivalent maternal body burden with long-term dosing (ng/kg of body weight)	13 ^c	25 ^c	19 ^d	39 ^d
Body burden from feed (ng/kg of body weight)	3	3	3	3
Total body burden (ng/kg of body weight)	16 ^e	28 ^e	22 ^e	42 ^e
EHMI (pg/kg of body weight per month)	237	423	330	630
Safety factor	3.2	9.6	3.2	9.6
PTMI (pg/kg of body weight per month)	74	44	103	66

^a Bolus dose (NOEL).^b Target maternal body burden from repeated dosing (LOEL).^c Assuming a linear relationship between fetal and maternal body burden (based on data in Table 9).^d Assumes a non-linear relationship between fetal and maternal body burden (based on data in Table 9).^e Assuming, for humans, a half-life of 7.6 years and 50% uptake from food (see equation on page 126).

provided the LOEL is 44pg/kg of body weight. As also shown in Table 14, use of the power model to extrapolate the maternal body burden with single doses to multiple doses would result in PTMIs of 103pg/kg of body weight for the NOEL and 66pg/kg of body weight for the LOEL. The range of PTMIs derived from the two studies, with either the linear or the power model to extrapolate the maternal body burden with single to multiple doses, is thus 40–100pg/kg of body weight per month. The Committee chose the mid-point of this range, 70pg/kg of body weight per month, as the PTMI. Furthermore, in accordance with the conclusions of the WHO consultation (5), the Committee concluded that this tolerable intake should be applied to intake of PCDDs, PCDFs and coplanar PCBs expressed as TEFs.

5.2.8.4 Comparison of PTMI with estimated intake from food

In the GEMS/Food regional diets, the range of estimated intake of toxic equivalents of PCDDs and PCDFs is 7–68pg/kg of body weight per month at the median and 15–160pg/kg of body weight per month at the 90th percentile of mean lifetime exposure, and that for coplanar PCBs is 7–57pg/kg of body weight per month at the median and 19–150pg/kg of body weight per month at the 90th percentile of consumption. The intakes estimated from national food consumption data were lower: 33–42pg/kg of body weight per month at the median and 81–100pg/kg of body weight per month at the 90th percentile for PCDDs and PCDFs, and 9–47pg/kg of body weight per month at the

median and 25–130 pg/kg of body weight per month at the 90th percentile for coplanar PCBs. Estimates could not be made for the sum of PCDDs, PCDFs and coplanar PCBs, because data on concentrations were submitted separately by countries.

The median and 90th percentile of the derived distribution of intakes were considered to describe long-term intake. A Monte Carlo calculation was used to predict these intakes for coplanar PCBs on the basis of two sets of distribution curves generated from information on mean concentrations in six major food groups and corresponding data on mean food consumption from several sources, by applying geometric standard deviations of 3 and 1.3 to the respective means. The geometric standard deviation for the food consumption curves accounted for long-term consumption patterns. As the mean intakes of the whole population tend not to change with the duration of a survey, use of mean consumer intakes to generate the curves for major food groups, rather than individual commodities, approximates the mean intakes of the whole population, as nearly all respondents were consumers.

5.2.8.5 Uncertainties

Several sources of uncertainty were identified in the data used to assess intake, which suggest that they are likely to be overestimates at both the median and the 90th percentile levels of consumption. Despite the uncertainties, the results suggest that a considerable fraction of the population will have a long-term mean intake above the PTMI.

Furthermore, despite the large amount of information on toxicity, substantial uncertainties remain which should be considered in applying the risk assessment and in interpreting the estimates of intake of PCDDs, PCDFs and coplanar PCBs. The Committee used the overall data to identify a level of intake of coplanar compounds in food that represents no appreciable risk to humans. The safety assessment includes adjustment for a number of uncertainties, including estimates of TEFs within orders of magnitude in order to relate the potency of 28 relatively poorly studied compounds to that of one well-studied compound, TCDD. Moreover, the relative proportion of TCDD and the other 28 compounds varies; TCDD typically constitutes a small percentage of the total toxic equivalents in foods.

The PTMI is not a limit of toxicity and does not represent a boundary between safe intake and intake associated with a significant increase in body burden or risk. Long-term intakes slightly above the PTMI would not necessarily result in adverse health effects but would erode the safety factor built into the calculations of the PTMI. It is not

possible, given current knowledge, to define the magnitude and duration of excess intake that would be associated with adverse health effects.

5.2.8.6 Effect of maximum limits on intake, risk and food availability

The concentrations of PCDDs, PCDFs and coplanar PCBs vary among foods. In establishing regulatory limits, the possible undesired consequences of their enforcement should be taken into account, such as reductions in the food supply. The Committee explored the theoretical effects of various maximum regulatory limits on compliance and on long-term average reduction of intake. On the basis of this analysis, the Committee concluded that, in order to achieve, for example, a 20% reduction in intake of coplanar compounds from food, the intake of a wide range of foods would have to be reduced by a similar percentage. This relationship exists because these contaminants are present at relatively high levels in major food types. Furthermore, in view of the half-times of these compounds in humans, setting regulatory limits on the basis of the PTMI would have no discernible effect on body burdens for several years.

In contrast, long-term reductions could be gained by identifying and eliminating the routes by which these compounds pass from the environment into food supplies. The Committee was informed that studies of environmental concentrations over time in several countries suggest that measures to control emissions to the environment generally have had a substantial impact on both the amounts of PCDDs and PCDFs present in the environment and the body burdens of the general public.

6. Future work

1. The Committee has been asked on several occasions to advise the Codex Committee on Food Additives and Contaminants on the relative risks associated with alternative proposed maximum limits for contaminants in foods. However, a maximum limit will not, in many cases, have a substantial effect on the long-term intake of the contaminant by the general population, nor will it have a measurable impact on public health unless a substantial proportion of the food supply is removed from the market. Nevertheless, maximum limits could have a positive influence on agricultural and industrial practices and contribute to reducing the intake of some contaminants for which the distribution is highly skewed. The Committee recommended more detailed consideration of this issue at a future meeting.

2. The Committee strongly reiterated its recommendation made at its fifty-fifth meeting for revision of the *Guide to specifications* (Annex 1, reference 100). This revision is urgently required, so that significant developments in methods of analysis can be included.
3. The Committee recommended continuation of its activity to update limits for heavy metals in food additives and concluded that acidity regulators and colours should be reviewed at its next meeting on food additives and contaminants.
4. The Committee recommended that the monograph that covers specifications for 16 modified starches should be divided into smaller monographs, as changes to one specification mean that the entire monograph must be changed.

7. Recommendations

1. In view of the large number of food additives and contaminants requiring evaluation or re-evaluation, the important role that the recommendations of the Committee play in the development of international food standards and of regulations in many countries, and the need for maintaining consistency and continuity within the Committee, it is strongly recommended that meetings of the Joint FAO/WHO Expert Committee on Food Additives continue to be held at least once yearly to evaluate these substances.
2. The Codex Alimentarius Commission has adopted International Numbering System (INS) numbers 472e and 472f for diacetyltartaric and fatty acid esters of glycerol and for tartaric, acetic and fatty acid esters of glycerol, mixed, respectively. At its fifty-first meeting (Annex 1, reference 137), the Committee established one specification under the name “diacetyltartaric and fatty acid esters of glycerol” to cover these two substances, and at the present meeting a temporary ADI was established. The specifications have been combined because, even if diacetyltartaric and fatty acid esters of glycerol and tartaric, acetic and fatty acid esters of glycerol, mixed, are manufactured from different raw materials, they meet all the criteria of the specifications and cannot be distinguished from each other by currently available analytical methods. The Committee recommended that the Codex Committee on Food Additives and Contaminants consider whether it would be more appropriate to have only one INS number for labelling purposes.
3. The Committee recognized that the revised “General specifications and recommendations for enzyme preparations used in food processing” in the *Compendium of food additive specifications, addendum 9* (FAO Food and Nutrition Paper, No. 52, Add. 9, 2001)

- contain many criteria for safety evaluations that are more appropriate for inclusion elsewhere. The Committee recommended that the project to update and consolidate principles and methods for the assessment of chemicals in food (see section 2.3) include the safety assessment of enzymes intended for use in food and a subsequent removal of these guidelines from the general specifications.
4. In view of the complexity of the analytical methods for determining PCDDs, PCDFs and coplanar PCBs, the Committee recommended that a specific validation protocol be developed. Laboratories involved in such analytical work should be encouraged by FAO/WHO to participate in collaborative studies and proficiency testing.
 5. A clear definition of “flavouring agent” has not been elaborated by the Committee, which recommended to FAO and WHO that such a definition be developed when updating principles for the assessment of chemicals in food.

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Annex 1

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152. *Evaluation of certain mycotoxins in food* (Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 906, 2002.
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Annex 2

Acceptable Daily Intakes, other toxicological information and information on specifications

Food additives evaluated toxicologically

Food additive	Specifications ^a	Acceptable daily intake (ADI in mg/kg of body weight) and other toxicological recommendations
Emulsifiers		
Diacetyltartaric and fatty acid esters of glycerol	R	0–50 (temporary) ^b
Tartaric, acetic and fatty acid esters of glycerol, mixed	W ^c	ADI “not limited” withdrawn ^c
Quillaia extracts	R, T ^b	0–5 (temporary) ^{b,d}
Enzyme preparation		
Invertase from <i>Saccharomyces cerevisiae</i>	N	Acceptable ^e
Food colours		
β-Carotene from <i>Blakeslea trispora</i>	N, T ^b	0–5 (group ADI) ^f
Curcumin	R	0–1 (temporary) ^b
Food salts		
Calcium dihydrogen diphosphate	N	Included in the maximum tolerable daily intake of 70 mg/kg of body weight for phosphates, diphosphates and polyphosphates
Monomagnesium phosphate	N, T ^b	
Sodium calcium polyphosphate	N	
Trisodium diphosphate	N, T ^b	
Glazing agent		
Hydrogenated poly-1-decene	R	0–6
Preservative		
Natamycin (pimaricin)	R, T ^b	0–0.3
Sweetening agent		
D-Tagatose	S	0–80
Thickening agents		
Carrageenan	R	ADI “not specified” ^g (group ADI) ^h
Processed <i>Eucheuma</i> seaweed	R	
Curdlan	R	ADI “not specified” ^g
Miscellaneous substances		
Acetylated oxidized starch	N, R ⁱ	ADI “not specified” ^g
α-Cyclodextrin	N	
Sodium sulfate	S	

^a N, new specifications prepared; R, existing specifications revised; S, specifications exist, revision not considered or required; T, the existing, new or revised specifications are tentative and new information is needed; W, existing specifications withdrawn.

^b See Annex 3.

- ^c The ADI was withdrawn because the specifications for tartaric, acetic and fatty acid esters of glycerol, mixed, were combined with those for diacetyltartaric and fatty acid esters of glycerol under the latter name at the fifty-first meeting (Annex 1, reference 137).
- ^d Applicable only to the unpurified extract.
- ^e Invertase from *Saccharomyces cerevisiae* that meets the specifications developed at the present meeting was considered to be acceptable because *S. cerevisiae* is commonly used in the preparation of food. Its use should be limited by good manufacturing practice.
- ^f Group ADI for β -carotene from *Blakeslea trispora* and synthetic β -carotene.
- ^g ADI "not specified" is used to refer to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice, i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.
- ^h Group ADI for carrageenan and processed *Eucheuma* seaweed.
- ⁱ The new specifications for acetylated oxidized starch were incorporated into the revised specifications for modified starches.

Food additives considered for specifications only

Food additive	Specification ^a
Acesulfame K (potassium salt)	R
Blackcurrant extract	R
D,L-Malic acid	R
Oxystearin	W
Pectins	R
Smoke flavourings	R
Tagetes extract	R

^a R, existing specifications revised; W, existing specifications withdrawn.

Flavouring agents evaluated by the Procedure for the Safety Evaluation of Flavouring Agents

Flavouring agent	No.	Specifications ^a	Conclusion based on current intake

Pyrazine derivatives

Structural class II

2-Methylpyrazine	761	N	No safety concern
2-Ethylpyrazine	762	N	
2-Propylpyrazine	763	N	
2-Isopropylpyrazine	764	N	
2,3-Dimethylpyrazine	765	N	
2,5-Dimethylpyrazine	766	N	
2,6-Dimethylpyrazine	767	N	

Flavouring agent	No.	Specifications ^a	Conclusion based on current intake
2-Ethyl-3-methylpyrazine	768	N	No safety concern
2-Ethyl-6-methylpyrazine	769	N	
2-Ethyl-5-methylpyrazine	770	N	
2,3-Diethylpyrazine	771	N	
2-Methyl-5-isopropylpyrazine	772	N	
2-Isobutyl-3-methylpyrazine	773	N	
2,3,5-Trimethylpyrazine	774	N	
2-Ethyl-3,(5 or 6)-dimethylpyrazine	775	N	
3-Ethyl-2,6-dimethylpyrazine	776	N	
2,3-Diethyl-5-methylpyrazine	777	N	
2,5-Diethyl-3-methylpyrazine	778	N	
3,5-Diethyl-2-methylpyrazine	779	N	
2,3,5,6-Tetramethylpyrazine	780	N	
5-Methyl-6,7-dihydro-5 <i>H</i> -cyclopentapyrazine	781	N	
6,7-Dihydro-2,3-dimethyl-5 <i>H</i> -cyclopentapyrazine	782	N	
Acetylpyrazine	784	N	
2-Acetyl-3-ethylpyrazine	785	N	
2-Acetyl-3,(5 or 6)-dimethylpyrazine	786	N	
Methoxypyrazine	787	N	
(2 or 5 or 6)-Methoxy-3-methylpyrazine	788	N	
2-Ethyl-(3 or 5 or 6)-methoxypyrazine	789	N	
2-Methoxy-(3 or 5 or 6)-isopropylpyrazine	790	N	
2-Methoxy-3-(1-methylpropyl)pyrazine	791	N	
2-Isobutyl-3-methoxypyrazine	792	N	
2-Acetyl-3-methylpyrazine	950	N	
Structural class III			
(Cyclohexylmethyl)pyrazine	783	N	No safety concern
2-Methyl-(3 or 5 or 6)-ethoxypyrazine	793	N	
2-(Mercaptomethyl)pyrazine	794	N	
2-Pyrazinylethane thiol	795	N	
Pyrazinylmethyl methyl sulfide	796	N	
(3 or 5 or 6)-(Methylthio)-2-methylpyrazine	797	N	
5-Methylquinoxaline	798	N	
Pyrazine	951	N	
5,6,7,8-Tetrahydroquinoxaline	952	N	
Aromatic substituted secondary alcohols, ketones and related esters			
Structural class I			
α -Methylbenzyl alcohol ^b	799	N	No safety concern
α -Methylbenzyl formate	800	N	
α -Methylbenzyl acetate	801	N	
α -Methylbenzyl propionate	802	N	
α -Methylbenzyl butyrate	803	N	
α -Methylbenzyl isobutyrate	804	N	
<i>p</i> . α -Dimethylbenzyl alcohol	805	N	

Flavouring agent	No.	Specifications ^a	Conclusion based on current intake
Acetophenone	806	N	No safety concern
4-Methylacetophenone	807	N	
<i>p</i> -Isopropylacetophenone	808	N	
2,4-Dimethylacetophenone	809	N	
Acetanisole	810	N	
1-(<i>p</i> -Methoxyphenyl)-2-propanone	813	N	
α -Methylphenethyl butyrate	814	N, T	
4-Phenyl-2-butanol	815	N	
4-Phenyl-2-butyl acetate	816	N	
4-(<i>p</i> -Tolyl)-2-butanone	817	N, T	
4-(<i>p</i> -Methoxyphenyl)-2-butanone	818	N	
4-Phenyl-3-buten-2-ol	819	N	
4-Phenyl-3-buten-2-one	820	N	
3-Methyl-4-phenyl-3-buten-2-one	821	N	
1-Phenyl-1-propanol	822	N	
α -Ethylbenzyl butyrate	823	N	
Propiophenone	824	N	
α -Propylphenethyl alcohol	825	N	
1-(<i>p</i> -Methoxyphenyl)-1-penten-3-one	826	N	
Ethyl benzoylacetate	834	N	
Ethyl 2-acetyl-3-phenylpropionate	835	N	
Structural class II			
4-Acetal-6- <i>tert</i> -butyl-1,1-dimethylindan	812	N	Additional data required
α -Isobutylphenethyl alcohol	827	N	No safety concern
4-Methyl-1-phenyl-2-pentanone	828	N	
1-(4-Methoxyphenyl)-4-methyl-1-penten-3-one	829	N	
3-Benzyl-4-heptanone	830	N	
1-Phenyl-1,2-propanedione	833	N	
Structural class III			
Methyl β -naphthyl ketone	811	N	No safety concern
Benzophenone	831	N	
1,3-Diphenyl-2-propanone	832	N	
Benzoin	836	N	

Flavouring agent	No.	Specifications ^a	Conclusion based on current intake
Benzyl acetoacetate	848	N	No safety concern
Benzyl benzoate ^c	24	R	
Benzyl phenylacetate	849	N	
Benzaldehyde ^c	22	R	
Benzaldehyde dimethyl acetal	837	N	
Benzaldehyde glyceryl acetal	838	N	
Benzaldehyde propylene glycol acetal	839	N	
Benzoic acid ^c	850	N	Evaluation not finalized ^d
Methyl benzoate	851	N	No safety concern
Ethyl benzoate	852	N	
Propyl benzoate	853	N	
Hexyl benzoate	854	N	
Isopropyl benzoate	855	N	
Isobutyl benzoate	856	N	
Isoamyl benzoate	857	N	
<i>cis</i> -3-Hexenyl benzoate	858	N	
Linalyl benzoate	859	N	
Geranyl benzoate	860	N	Evaluation not finalized ^d
Glyceryl tribenzoate	861	N, T	
Propylene glycol dibenzoate	862	N, T	
Methylbenzyl acetate (mixed <i>ortho</i> -, <i>meta</i> - and <i>para</i> -isomers)	863	N	No safety concern
<i>p</i> -Isopropylbenzyl alcohol	864	N	
4-Ethylbenzaldehyde	865	N	
Tolualdehydes (mixed <i>ortho</i> -, <i>meta</i> - and <i>para</i> -isomers)	866	N, T	
Tolualdehyde glyceryl acetal	867	N	
Cuminaldehyde	868	N	
2,4-Dimethylbenzaldehyde	869	N	
Benzyl 2-methoxyethyl acetal	840	N	

Hydroxy- and alkoxy-substituted benzyl derivatives

Structural class I

4-Hydroxybenzyl alcohol	955	— ^e	No safety concern
4-Hydroxybenzaldehyde	956	— ^e	
4-Hydroxybenzoic acid	957	— ^e	
2-Hydroxybenzoic acid	958	— ^e	
Butyl <i>p</i> -hydroxybenzoate	870	N, T	Evaluation not finalized ^d
Anisyl alcohol	871	N	No safety concern
Anisyl formate	872	N, T	
Anisyl acetate	873	N	
Anisyl propionate	874	N	
Anisyl butyrate	875	N	
Anisyl phenylacetate	876	N	

Flavouring agent	No.	Specifications ^a	Conclusion based on current intake
Veratraldehyde	877	N	No safety concern
<i>p</i> -Methoxybenzaldehyde	878	N	
<i>p</i> -Ethoxybenzaldehyde	879	N	
Methyl <i>o</i> -methoxybenzoate	880	N	
2-Methoxybenzoic acid	881	N	
3-Methoxybenzoic acid	882	N	
4-Methoxybenzoic acid	883	N	
Methyl anisate	884	N	
Ethyl <i>p</i> -anisate	885	N	
Vanillyl alcohol	886	N	
Vanillin ^f	889	N	
4-Hydroxy-3-methoxybenzoic acid	959	— ^e	
Vanillin acetate	890	N	
Vanillin isobutyrate	891	N	
Salicylaldehyde	897	N	
2-Hydroxy-4-methylbenzaldehyde	898	N	
Methyl salicylate ^g	899	N	
Ethyl salicylate	900	N	
Butyl salicylate	901	N	
Isobutyl salicylate	902	N	
Isoamyl salicylate	903	N	
Benzyl salicylate	904	N	
Phenethyl salicylate	905	N	
<i>o</i> -Tolyl salicylate	907	N	
2,4-Dihydroxybenzoic acid	908	N	
Structural class II			
Vanillyl ethyl ether	887	N	No safety concern
Vanillyl butyl ether	888	N	
Ethyl vanillin ^h	893	N	
Vanillin <i>erythro</i> - and <i>threo</i> -butan-2,3-diol acetal	960	— ^e	
Ethyl vanillin isobutyrate	953	N	
Ethyl vanillin propylene glycol acetal	954	N, T	
Piperonyl acetate	894	N	
Piperonyl isobutyrate	895	N	
Piperonal ⁱ	896	N	
Ethyl vanillin β-D-glucopyranoside	892	N	
Aliphatic acyclic diols, triols and related substances			
Structural class I			
Glycerol ^j	909	N, T	Evaluation not finalized ^d
1,2,3-Tris[(1'-ethoxy)ethoxy]propane	913	N	No safety concern
Glyceryl monostearate	918	N, T	Evaluation not finalized ^d
Glyceryl monooleate	919	N, T	
Triacetin	920	N, T	
Glyceryl tripropanoate	921	N, T	

Flavouring agent	No.	Specifications ^a	Conclusion based on current intake
Tributyrin	922	N, T	Evaluation not finalized ^d
Glycerol 5-hydroxydecanoate	923	N, T	
Glycerol 5-hydroxydodecanoate	924	N, T	
Propylene glycol ^k	925	N, T	
Propylene glycol stearate	926	N, T	
1,2-Di[(1-ethoxy)ethoxy]propane	927	N	No safety concern
Lactic acid	930	N	
Ethyl lactate ^l	931	N	
Butyl lactate	932	N	
Potassium 2-(1'-ethoxy)ethoxypropanoate	933	N	
<i>cis</i> -3-Hexenyl lactate	934	N	
Butyl butyryllactate	935	N	
Pyruvic acid	936	N	
Pyruvaldehyde	937	N, T	
Ethyl pyruvate	938	N	
Isoamyl pyruvate	939	N	
Structural class III			
3-Oxohexanoic acid glyceride	910	N, T	No safety concern
3-Oxooctanoic acid glyceride	911	N, T	
Heptanal glyceryl acetal (mixed 1,2 and 1,3 acetals)	912	N	
3-Oxodecanoic acid glyceride	914	N, T	Evaluation not finalized ^d
3-Oxododecanoic acid glyceride	915	N, T	
3-Oxotetradecanoic acid glyceride	916	N, T	
3-Oxohexadecanoic acid glyceride	917	N, T	
4-Methyl-2-pentyl-1,3-dioxolane	928	N	No safety concern
2,2,4-Trimethyl-1,3-oxacyclopentane	929	N	
Aliphatic acyclic acetals			
Structural class I			
1,1-Dimethoxyethane	940	N	No safety concern
Acetal	941	N	
Heptanal dimethyl acetal	947	N	
4-Heptenal diethyl acetal	949	N	
Octanal dimethyl acetal	942	N	
2,6-Nonadienal diethyl acetal	946	N	
Decanal dimethyl acetal	945	N	
Citral dimethyl acetal	944	N	
Citral diethyl acetal	948	N	
Acetaldehyde ethyl <i>cis</i> -3-hexenyl acetal	943	N, T	

^a N, new specifications prepared; R, existing specifications revised; T, the existing, new or revised specifications are tentative and new information is needed.

^b An ADI of 0–0.1 mg/kg of body weight was established for α -methylbenzyl alcohol by the Committee at its forty-first meeting (WHO Technical Report Series, No. 837, 1993), which was maintained at the present meeting.

^c A group ADI of 0–5 mg/kg of body weight for benzoic acid, the benzoate salts (calcium, potassium and sodium), benzaldehyde, benzyl acetate and benzyl alcohol, expressed as

benzoic acid equivalents, was confirmed by the Committee at its forty-sixth meeting (WHO Technical Report Series, No. 868, 1997) and extended to include benzyl benzoate at the present meeting.

^d Further information is required to determine whether this substance is in current use as a flavouring agent.

^e Specifications will be considered at the fifty-ninth meeting of the Committee.

^f An ADI of 0–10 mg/kg of body weight was established for vanillin by the Committee at its eleventh meeting (WHO Technical Report Series, No. 383, 1968), which was maintained at the present meeting.

^g An ADI of 0–0.5 mg/kg of body weight was established for methyl salicylate by the Committee at its eleventh meeting (WHO Technical Report Series, No. 383, 1968), which was maintained at the present meeting.

^h An ADI of 0–3 mg/kg of body weight was established for ethyl vanillin by the Committee at its forty-fourth meeting (WHO Technical Report Series, No. 859, 1995), which was maintained at the present meeting.

ⁱ An ADI of 0–2.5 mg/kg of body weight was established for piperonal by the Committee at its eleventh meeting (WHO Technical Report Series, No. 383, 1968), which was maintained at the present meeting.

^j An ADI “not specified” was established for glycerol by the Committee at its twentieth meeting (WHO Technical Report Series, No. 599, 1976), which was maintained at the present meeting.

^k An ADI of 0–25 mg/kg of body weight was established for propylene glycol by the Committee at its seventeenth meeting (WHO Technical Report Series, No. 539, 1974), which was maintained at the present meeting.

^l Ethyl lactate was included in the group ADI “not specified” for lactic acid and its salts that was established by the Committee at its twenty-sixth meeting (WHO Technical Report Series, No. 683, 1982), which was maintained at the present meeting.

Flavouring agents considered for specifications only

Flavouring agent	No.	Specifications ^a
Allyl tiglate	10	R
Allyl cyclohexane acetate	12	R
Allyl cyclohexane butyrate	14	R
Allyl cyclohexane valerate	15	R
Allyl cyclohexane hexanoate	16	R
Isoamyl formate	42	R
Isoamyl 2-methylbutyrate	51	R
Geranyl acetate	58	R
Rhodiny l propionate	64	R
Geranyl hexanoate	70	R
Geranyl isobutyrate	72	R
Rhodiny l isobutyrate	74	R
Rhodiny l isovalerate	77	R
3,7-Dimethyl-2,6-octadien-1-yl 2-ethylbutanoate	78	R
Heptanal	95	R
Nonanal	101	R
Undecanal	107	R
Lauric acid	111	R, T
Myristic acid	113	R, T
Palmitic acid	115	R, T
Stearic acid	116	R, T
Propyl formate	117	R
<i>n</i> -Amyl formate	119	R
Isobutyl formate	124	R
<i>n</i> -Amyl heptanoate	170	R

Flavouring agent	No.	Specifications ^a
Isobutyl heptanoate	172	R
Nonyl octanoate	178	R
Methyl laurate	180	R
Isoamyl laurate	182	R, T
Butyl stearate	184	R
<i>trans</i> -3-Heptenyl 2-methyl propanoate	191	R
Methyl 2-methylbutyrate	205	R
2-Methylbutyl 2-methylbutyrate	212	R
ω -6-Hexadecenlactone	240	R
<i>cis</i> -4-Hydroxy-6-dodecenoic acid lactone	249	R
2-Methylpentanal	260	R
2-Methylhexanoic acid	265	R
5-Methylhexanoic acid	266	R
2-Methyloctanal	270	R
2,6-Dimethyloctanal	273	R
2-Methylundecanal	275	R
Isopropyl formate	304	R
Isopropyl propionate	306	R
Isopropyl hexanoate	308	R
<i>cis</i> -5-Octen-1-ol	322	R
<i>cis</i> -5-Octenal	323	R
<i>cis</i> -6-Nonenal	325	R
4-Decenal	326	R
9-Decenoic acid	328	R
10-Undecenal	330	R
Methyl 3-hexenoate	334	R
Butyl 10-undecenoate	344	R
2-Methyl-3-pentenoic acid	347	R
2,6-Dimethyl-6-hepten-1-ol	348	R
Ethyl 2-methyl-3-pentenoate	350	R
Hexyl 2-methyl-3(4)-pentenoate (mixture)	352	R
Terpinyl formate	367	R
Terpinyl butyrate	370	R
Terpinyl isovalerate	372	R
<i>p</i> -Menth-8-en-1-ol	374	R
α -Ionone	388	R
γ -Ionone	390	R, T
Allyl α -ionone	401	R
α -iso-Methylionone	404	R
5-Hydroxy-4-octanone	416	R
2-Hydroxy-2-cyclohexen-1-one	424	R
(+)-Neo-menthol	428	R
<i>p</i> -Menth-1-en-3-ol	434	R
2-Ethyl-1,3,3-trimethyl-2-norbornanol	440	R
Methyl 1-acetoxycyclohexyl ketone	442	R
1-Ethylhexyl tiglate	448	R
(1-Buten-1-yl) methyl sulfide	457	R
3-(Methylthio)propanol	461	R
3-(Methylthio)propyl acetate	478	R
Allyl thiopropionate	490	R, T

Flavouring agent	No.	Specifications ^a
2-Propanethiol	510	R
2-Naphthalenethiol	531	R
Trithioacetone	543	R
2,5-Dimethyl-2,5-dihydroxy-1,4-dithiane	562	R
2-Methyl-2-(methyldithio)propanal	580	R
Ethyl 2-(methyldithio)propionate	581	R
Methyl 2-oxo-3-methylpentanoate	591	R
Geranyl acetoacetate	599	R
3-(Hydroxymethyl)-2-heptanone	604	R, T
1,4-Nonanediol diacetate	609	R, T
Aconitic acid	627	R, T
3-Phenylpropyl hexanoate	642	R, T
3-Phenylpropionaldehyde	645	R
Cinnamaldehyde ethylene glycol acetal	648	R
Cinnamyl butyrate	652	R
Cinnamaldehyde	656	R
Propyl cinnamate	660	R
Butyl cinnamate	663	R
Heptyl cinnamate	666	R
Phenethyl cinnamate	671	R
3-Phenylpropyl cinnamate	672	R
Cinnamyl cinnamate	673	R
α -Amylcinnamyl formate	676	R
α -Amylcinnamyl acetate	677	R
α -Amylcinnamyl isovalerate	678	R, T
α -Amylcinnamaldehyde dimethyl acetal	681	R
<i>o</i> -Tolyl acetate	698	R
<i>p</i> -Vinylphenol	711	R
Guaiacyl phenylacetate	719	R
Hydroquinone monoethyl ether	720	R
4-Ethyl-2,6-dimethoxyphenol	723	R
4-Propyl-2,6-dimethoxyphenol	724	R
4-Allyl-2,6-dimethoxyphenol	726	R
Dihydroxyacetophenone	729	R, T
Vanillylidene acetone	732	R
Furfuryl propionate	740	R
Furfuryl pentanoate	741	R
Furfuryl octanoate	742	R
Furfuryl 3-methylbutanoate	743	R
Amyl 2-furoate	748	R
Hexyl 2-furoate	749	R
Octyl 2-furoate	750	R
2-Phenyl-3-carboethoxyfuran	752	R, T
Furfuryl butyrate	759	R
Cinnamyl benzoate	760	R

^a R, existing specifications revised; T, the existing, new or revised specifications are tentative and new information is required.

Contaminants

Contaminant	Tolerable intake and other toxicological recommendations
3-Chloro-1,2-propanediol	Provisional maximum tolerable daily intake: 2 µg/kg of body weight
1,3-Dichloro-2-propanol	Establishment of a tolerable intake was considered to be inappropriate because of the nature of the toxicity observed (tumorigenic in various organs in rats and interacts with chromosomes and/or DNA); the Committee noted that the dose that caused tumours in rats (19 mg/kg of body weight per day) was about 20 000 times the highest estimated intake of 1,3-dichloro-2-propanol by consumers of soya sauce (1 µg/kg of body weight per day).
Polychlorinated dibenzodioxins, polychlorinated dibenzofurans and coplanar polychlorinated biphenyls	Provisional tolerable monthly intake: 70 pg/kg of body weight

Annex 3

Further information required or desired

Toxicological information

Diacetyltartaric and fatty acid esters of glycerol

The following information relating to the 2-year study on toxicity in rats is required for evaluation in 2003:

1. In order to determine whether some of the adverse effects that were observed were treatment-related, the groups treated with diacetyltartaric and fatty acid esters of glycerol should be compared with both untreated and monoglyceride-treated controls, and the control groups should be compared with one another.
2. Additional information on the incidence of myocardial fibrosis and adrenal medullary hyperplasia in animals at the lowest and intermediate doses should be provided.

Curcumin

The results of a study on reproductive toxicity with a substance complying with the specifications for curcumin, known to be in progress, is required for evaluation in 2003.

Information on specifications

β -Carotene from *Blakeslea trispora*

Information is required on the method of analysis for residual solvents (ethyl acetate and isobutyl acetate). This information is required for evaluation in 2003.

Monomagnesium phosphate and trisodium diphosphate

Information is required on the loss on drying, loss on ignition, test method for loss on ignition and assay method for the hydrates. This information is required for evaluation in 2003.

Natamycin

Information is required on the level and determination of water content, limit for lead, specific rotation, assay value and method of assay for the commercial product. Comments on other aspects of the monograph are invited. This information is required for evaluation in 2003.

Quillaia extracts

The existing specifications for quillaia extracts were revised in order to clarify the differences between unpurified and semi-purified extracts. As additional information on composition (minimum and

maximum percentages of saponins in unpurified and semi-purified extracts) is necessary, the specifications were designated as tentative. Once the requested information has been received, the Committee will consider whether separate specifications for unpurified and semi-purified extracts are required. This information is required for evaluation in 2003.

SELECTED WHO PUBLICATIONS OF RELATED INTEREST

Evaluation of certain mycotoxins in food.

Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives.

WHO Technical Report Series, No. 906, 2002 (70 pages)

Safety evaluation of certain mycotoxins in food.

WHO Food Additives Series, No. 47, 2001 (707 pages)

Evaluation of certain food additives and contaminants.

Fifty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives.

WHO Technical Report Series, No. 901, 2001 (117 pages)

Safety evaluation of certain food additives and contaminants.

WHO Food Additives Series, No. 46, 2001 (392 pages)

Evaluation of certain food additives and contaminants.

Fifty-third report of the Joint FAO/WHO Expert Committee on Food Additives.

WHO Technical Report Series, No. 896, 2000 (136 pages)

Safety evaluation of certain food additives and contaminants.

WHO Food Additives Series, No. 44, 2000 (539 pages)

Evaluation of certain food additives.

Fifty-first report of the Joint FAO/WHO Expert Committee on Food Additives.

WHO Technical Report Series, No. 891, 2000 (176 pages)

Safety evaluation of certain food additives.

WHO Food Additives Series, No. 42, 1999 (494 pages)

Evaluation of certain food additives and contaminants.

Forty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives.

WHO Technical Report Series, No. 884, 1999 (104 pages)

This report represents the conclusions of a Joint FAO/WHO Expert Committee convened to evaluate the safety of various food additives and contaminants, with a view to recommending Acceptable Daily Intakes (ADIs) and tolerable intakes, respectively, and to prepare specifications for the identity and purity of food additives.

The first part of the report contains a general discussion of the principles governing the toxicological evaluation of food additives (including flavouring agents) and contaminants, assessments of intake, and the establishment and revision of specifications for food additives. A summary follows of the Committee's evaluations of toxicological and intake data on various specific food additives (diacetyltartaric and fatty acid esters of glycerol, quillaia extracts, invertase from *Saccharomyces cerevisiae*, β -carotene from *Blakeslea trispora*, curcumin, phosphates, diphosphates and polyphosphates, hydrogenated poly-1-decene, natamycin, D-tagatose, carrageenan, processed *Eucheuma* seaweed, curdlan, acetylated oxidized starch, α -cyclodextrin and sodium sulfate), flavouring agents and contaminants (3-chloro-1,2-propanediol, 1,3-dichloro-2-propanol, and a large number of polychlorinated dibenzodioxins, polychlorinated dibenzofurans and coplanar polychlorinated biphenyls). Annexed to the report are tables summarizing the Committee's recommendations for ADIs of the food additives and tolerable intakes of the contaminants considered, changes in the status of specifications of these food additives and specific flavouring agents, and further information required or desired.

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SAFETY DATA SHEET

according to Regulation (EC) No. 1907/2006

Version 7.1

Revision Date 16.02.2023

Print Date 27.02.2023

GENERIC EU MSDS - NO COUNTRY SPECIFIC DATA - NO OEL DATA

SECTION 1: Identification of the substance/mixture and of the company/undertaking**1.1 Product identifiers**

Product name : 2,3,5,6-Tetramethylpyrazine

Product Number : 183938

Brand : Aldrich

REACH No. : A registration number is not available for this substance as the substance or its uses are exempted from registration, the annual tonnage does not require a registration or the registration is envisaged for a later registration deadline.

CAS-No. : 1124-11-4

1.2 Relevant identified uses of the substance or mixture and uses advised against

Identified uses : Laboratory chemicals, Manufacture of substances

1.3 Details of the supplier of the safety data sheet

Company : Sigma-Aldrich Chemie GmbH
Industriestrasse 25
CH-9471 BUCHS

Telephone : +41 81 755 2511

Fax : +41 81 756 5449

E-mail address : technischerservice@merckgroup.com

1.4 Emergency telephone

Emergency Phone # : +41 43-508-2011 (CHEMTREC)
+41 44-251-5151 (Tox-Zentrum)
145(Tox Info Suisse)

SECTION 2: Hazards identification**2.1 Classification of the substance or mixture****Classification according to Regulation (EC) No 1272/2008**

Acute toxicity, Oral (Category 4), H302

Skin irritation (Category 2), H315

Eye irritation (Category 2), H319

Specific target organ toxicity - single exposure (Category 3), Respiratory system, H335

For the full text of the H-Statements mentioned in this Section, see Section 16.

2.2 Label elements**Labelling according Regulation (EC) No 1272/2008**

Pictogram



Signal Word

Warning

Hazard statement(s)

H302

Harmful if swallowed.

H315

Causes skin irritation.

H319

Causes serious eye irritation.

H335

May cause respiratory irritation.

Precautionary statement(s)

P261

Avoid breathing dust/ fume/ gas/ mist/ vapors/ spray.

P264

Wash skin thoroughly after handling.

P270

Do not eat, drink or smoke when using this product.

P301 + P312

IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell.

P302 + P352

IF ON SKIN: Wash with plenty of water.

P305 + P351 + P338

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Supplemental Hazard Statements

none

Reduced Labeling (<= 125 ml)

Pictogram



Signal Word

Warning

Hazard statement(s)

none

Precautionary statement(s)

none

Supplemental Hazard Statements

none

2.3 Other hazards

This substance/mixture contains no components considered to be either persistent, bioaccumulative and toxic (PBT), or very persistent and very bioaccumulative (vPvB) at levels of 0.1% or higher.

SECTION 3: Composition/information on ingredients

3.1 Substances

Formula : C₈H₁₂N₂
Molecular weight : 136,19 g/mol
CAS-No. : 1124-11-4
EC-No. : 214-391-2

Component		Classification	Concentration
2,3,5,6-Tetramethylpyrazine			
CAS-No.	1124-11-4	Acute Tox. 4; Skin Irrit. 2; Eye Irrit. 2; STOT SE 3; H302, H315, H319, H335	<= 100 %
EC-No.	214-391-2		



For the full text of the H-Statements mentioned in this Section, see Section 16.

SECTION 4: First aid measures

4.1 Description of first-aid measures

General advice

Show this material safety data sheet to the doctor in attendance.

If inhaled

After inhalation: fresh air.

In case of skin contact

In case of skin contact: Take off immediately all contaminated clothing. Rinse skin with water/ shower.

In case of eye contact

After eye contact: rinse out with plenty of water. Call in ophthalmologist. Remove contact lenses.

If swallowed

After swallowing: immediately make victim drink water (two glasses at most). Consult a physician.

4.2 Most important symptoms and effects, both acute and delayed

The most important known symptoms and effects are described in the labelling (see section 2.2) and/or in section 11

4.3 Indication of any immediate medical attention and special treatment needed

No data available

SECTION 5: Firefighting measures

5.1 Extinguishing media

Suitable extinguishing media

Water Foam Carbon dioxide (CO₂) Dry powder

Unsuitable extinguishing media

For this substance/mixture no limitations of extinguishing agents are given.

5.2 Special hazards arising from the substance or mixture

Carbon oxides

Nitrogen oxides (NO_x)

Combustible.

Development of hazardous combustion gases or vapours possible in the event of fire.

5.3 Advice for firefighters

Stay in danger area only with self-contained breathing apparatus. Prevent skin contact by keeping a safe distance or by wearing suitable protective clothing.

5.4 Further information

Suppress (knock down) gases/vapors/mists with a water spray jet. Prevent fire extinguishing water from contaminating surface water or the ground water system.



SECTION 6: Accidental release measures

6.1 Personal precautions, protective equipment and emergency procedures

Advice for non-emergency personnel: Avoid inhalation of dusts. Avoid substance contact. Ensure adequate ventilation. Evacuate the danger area, observe emergency procedures, consult an expert.
For personal protection see section 8.

6.2 Environmental precautions

Do not let product enter drains.

6.3 Methods and materials for containment and cleaning up

Cover drains. Collect, bind, and pump off spills. Observe possible material restrictions (see sections 7 and 10). Take up dry. Dispose of properly. Clean up affected area. Avoid generation of dusts.

6.4 Reference to other sections

For disposal see section 13.

SECTION 7: Handling and storage

7.1 Precautions for safe handling

For precautions see section 2.2.

7.2 Conditions for safe storage, including any incompatibilities

Storage conditions

Tightly closed. Dry.

Storage class

Storage class (TRGS 510): 11: Combustible Solids

7.3 Specific end use(s)

Apart from the uses mentioned in section 1.2 no other specific uses are stipulated

SECTION 8: Exposure controls/personal protection

8.1 Control parameters

Ingredients with workplace control parameters

8.2 Exposure controls

Personal protective equipment

Eye/face protection

Use equipment for eye protection tested and approved under appropriate government standards such as NIOSH (US) or EN 166(EU). Safety glasses

Skin protection

Handle with gloves. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use in accordance with applicable laws and good laboratory practices. Wash and dry hands.

The selected protective gloves have to satisfy the specifications of Regulation (EU) 2016/425 and the standard EN 374 derived from it.

Full contact

Material: Nitrile rubber



Minimum layer thickness: 0,11 mm
Break through time: 480 min
Material tested: Dermatril® (KCL 740 / Aldrich Z677272, Size M)

Splash contact
Material: Nitrile rubber
Minimum layer thickness: 0,11 mm
Break through time: 480 min
Material tested: Dermatril® (KCL 740 / Aldrich Z677272, Size M)

data source: KCL GmbH, D-36124 Eichenzell, phone +49 (0)6659 87300, e-mail sales@kcl.de, test method: EN374

If used in solution, or mixed with other substances, and under conditions which differ from EN 374, contact the supplier of the EC approved gloves. This recommendation is advisory only and must be evaluated by an industrial hygienist and safety officer familiar with the specific situation of anticipated use by our customers. It should not be construed as offering an approval for any specific use scenario.

Body Protection

protective clothing

Respiratory protection

required when dusts are generated.

Our recommendations on filtering respiratory protection are based on the following standards: DIN EN 143, DIN 14387 and other accompanying standards relating to the used respiratory protection system.

Recommended Filter type: Filter type P2

The entrepreneur has to ensure that maintenance, cleaning and testing of respiratory protective devices are carried out according to the instructions of the producer.

These measures have to be properly documented.

Control of environmental exposure

Do not let product enter drains.

SECTION 9: Physical and chemical properties

9.1 Information on basic physical and chemical properties

a) Physical state	solid
b) Color	No data available
c) Odor	No data available
d) Melting point/freezing point	Melting point/range: 77 - 80 °C - lit.
e) Initial boiling point and boiling range	190 °C - lit.
f) Flammability (solid, gas)	No data available
g) Upper/lower flammability or explosive limits	No data available
h) Flash point	Not applicable



i) Autoignition temperature	No data available
j) Decomposition temperature	No data available
k) pH	No data available
l) Viscosity	Viscosity, kinematic: No data available Viscosity, dynamic: No data available
m) Water solubility	No data available
n) Partition coefficient: n-octanol/water	No data available
o) Vapor pressure	No data available
p) Density	1,08 g/cm ³ at 20 °C
Relative density	No data available
q) Relative vapor density	No data available
r) Particle characteristics	No data available
s) Explosive properties	No data available
t) Oxidizing properties	none

9.2 Other safety information

No data available

SECTION 10: Stability and reactivity

10.1 Reactivity

The following applies in general to flammable organic substances and mixtures: in correspondingly fine distribution, when whirled up a dust explosion potential may generally be assumed.

10.2 Chemical stability

The product is chemically stable under standard ambient conditions (room temperature) .

10.3 Possibility of hazardous reactions

Violent reactions possible with:
Strong acids
strong oxidising agents

10.4 Conditions to avoid

no information available

10.5 Incompatible materials

No data available

10.6 Hazardous decomposition products

In the event of fire: see section 5



SECTION 11: Toxicological information

11.1 Information on toxicological effects

Acute toxicity

LD50 Oral - Rat - 1.910 mg/kg

Remarks: (RTECS)

Inhalation: No data available

Dermal: No data available

Skin corrosion/irritation

Remarks: No data available

Serious eye damage/eye irritation

Remarks: No data available

Respiratory or skin sensitization

No data available

Germ cell mutagenicity

No data available

Carcinogenicity

No data available

Reproductive toxicity

No data available

Specific target organ toxicity - single exposure

May cause respiratory irritation.

Specific target organ toxicity - repeated exposure

No data available

Aspiration hazard

No data available

11.2 Additional Information

Endocrine disrupting properties

Product:

Assessment

The substance/mixture does not contain components considered to have endocrine disrupting properties according to REACH Article 57(f) or Commission Delegated regulation (EU) 2017/2100 or Commission Regulation (EU) 2018/605 at levels of 0.1% or higher.

RTECS: UQ3905000

To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated.

Other dangerous properties can not be excluded.

Further data:

Handle in accordance with good industrial hygiene and safety practice.



SECTION 12: Ecological information

12.1 Toxicity

No data available

12.2 Persistence and degradability

No data available

12.3 Bioaccumulative potential

No data available

12.4 Mobility in soil

No data available

12.5 Results of PBT and vPvB assessment

This substance/mixture contains no components considered to be either persistent, bioaccumulative and toxic (PBT), or very persistent and very bioaccumulative (vPvB) at levels of 0.1% or higher.

12.6 Endocrine disrupting properties

Product:

Assessment : The substance/mixture does not contain components considered to have endocrine disrupting properties according to REACH Article 57(f) or Commission Delegated regulation (EU) 2017/2100 or Commission Regulation (EU) 2018/605 at levels of 0.1% or higher.

12.7 Other adverse effects

We have no quantitative data concerning the ecological effects of this product.
Further information on ecology
Discharge into the environment must be avoided.

SECTION 13: Disposal considerations

13.1 Waste treatment methods

Product

See www.retrologistik.com for processes regarding the return of chemicals and containers, or contact us there if you have further questions.

SECTION 14: Transport information

14.1 UN number

ADR/RID: -

IMDG: -

IATA: -

14.2 UN proper shipping name

ADR/RID: Not dangerous goods

IMDG: Not dangerous goods

IATA: Not dangerous goods

14.3 Transport hazard class(es)

ADR/RID: -

IMDG: -

IATA: -

14.4 Packaging group

ADR/RID: -

IMDG: -

IATA: -



14.5 Environmental hazards

ADR/RID: no

IMDG Marine pollutant: no

IATA: no

14.6 Special precautions for user

No data available

Further information

Not classified as dangerous in the meaning of transport regulations.

SECTION 15: Regulatory information

15.1 Safety, health and environmental regulations/legislation specific for the substance or mixture

This material safety data sheet complies with the requirements of Regulation (EC) No. 1907/2006.

Other regulations

Observe work restrictions regarding maternity protection in accordance to Dir 92/85/EEC or stricter national regulations where applicable.

Take note of Dir 94/33/EC on the protection of young people at work.

15.2 Chemical Safety Assessment

For this product a chemical safety assessment was not carried out

SECTION 16: Other information

Full text of H-Statements referred to under sections 2 and 3.

H302	Harmful if swallowed.
H315	Causes skin irritation.
H319	Harmful if swallowed.
H335	Causes skin irritation.



Full text of other abbreviations

ADN - European Agreement concerning the International Carriage of Dangerous Goods by Inland Waterways; ADR - Agreement concerning the International Carriage of Dangerous Goods by Road; AIIC - Australian Inventory of Industrial Chemicals; ASTM - American Society for the Testing of Materials; bw - Body weight; CMR - Carcinogen, Mutagen or Reproductive Toxicant; DIN - Standard of the German Institute for Standardisation; DSL - Domestic Substances List (Canada); ECx - Concentration associated with x% response; ELx - Loading rate associated with x% response; EmS - Emergency Schedule; ENCS - Existing and New Chemical Substances (Japan); ErCx - Concentration associated with x% growth rate response; GHS - Globally Harmonized System; GLP - Good Laboratory Practice; IARC - International Agency for Research on Cancer; IATA - International Air Transport Association; IBC - International Code for the Construction and Equipment of Ships carrying Dangerous Chemicals in Bulk; IC50 - Half maximal inhibitory concentration; ICAO - International Civil Aviation Organization; IECSC - Inventory of Existing Chemical Substances in China; IMDG - International Maritime Dangerous Goods; IMO - International Maritime Organization; ISHL - Industrial Safety and Health Law (Japan); ISO - International Organisation for Standardization; KECI - Korea Existing Chemicals Inventory; LC50 - Lethal Concentration to 50 % of a test population; LD50 - Lethal Dose to 50% of a test population (Median Lethal Dose); MARPOL - International Convention for the Prevention of Pollution from Ships; n.o.s. - Not Otherwise Specified; NO(A)EC - No Observed (Adverse) Effect Concentration; NO(A)EL - No Observed (Adverse) Effect Level; NOELR - No Observable Effect Loading Rate; NZIoC - New Zealand Inventory of Chemicals; OECD - Organization for Economic Co-operation and Development; OPPTS - Office of Chemical Safety and Pollution Prevention; PBT - Persistent, Bioaccumulative and Toxic substance; PICCS - Philippines Inventory of Chemicals and Chemical Substances; (Q)SAR - (Quantitative) Structure Activity Relationship; REACH - Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals; RID - Regulations concerning the International Carriage of Dangerous Goods by Rail; SADT - Self-Accelerating Decomposition Temperature; SDS - Safety Data Sheet; TCSI - Taiwan Chemical Substance Inventory; TECI - Thailand Existing Chemicals Inventory; TSCA - Toxic Substances Control Act (United States); UN - United Nations; UNRTDG - United Nations Recommendations on the Transport of Dangerous Goods; vPvB - Very Persistent and Very Bioaccumulative

Further information

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. The information in this document is based on the present state of our knowledge and is applicable to the product with regard to appropriate safety precautions. It does not represent any guarantee of the properties of the product. Sigma-Aldrich Corporation and its Affiliates shall not be held liable for any damage resulting from handling or from contact with the above product. See www.sigma-aldrich.com and/or the reverse side of invoice or packing slip for additional terms and conditions of sale.

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