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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.

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

Contents

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; LD_{50}, 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:

a) unsubstituted pyrazine (No. 40);
b) pyrazine derivatives containing a hydrocarbon (alkyl, alicyclic or alkylaryl) substituent (Nos 1–22 and Nos 33, 39 and 41);
c) pyrazine derivatives containing an oxygenated functional group and aliphatic side chain (Nos 23–32, 34);
d) 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 compounds), 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).

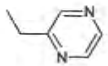
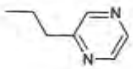
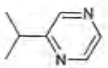
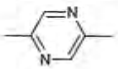
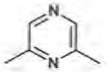
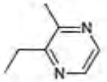
The total annual volume of use of pyrazine and quinoxaline derivatives as flavoring ingredients is 2135 kg in the USA (Lucas et al., 1999). Approximately two-thirds of the total annual volume arises from use of three substances (2,3,5-trimethylpyrazine (No. 14), 347 kg; 2,3,5,6-tetramethylpyrazine (No. 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 intake[1] ("eaters only") of greater than 3 μg/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 (μg/kg) calculated as follows: (((annual volume, kg)×(1×10^9 μg/kg))/(population×survey correction factor×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 (μg/kg body weight per day) calculated as follows: ((μg/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

(*Continued on next page*)

Table 1 (*continued*)

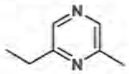
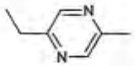
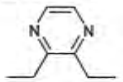
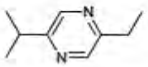
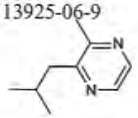
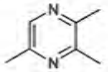
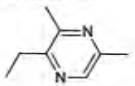
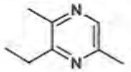
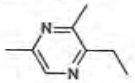
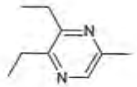
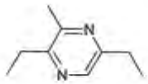
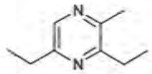
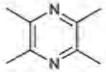
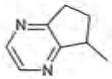
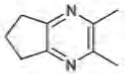
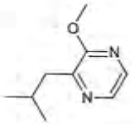
Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume (kg)	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods (kg)	Consumption ratio
				μg/day	μg/kg bw/day		
9. 2-Ethyl-6-methylpyrazine	3919	13925-03-6 	3	0.4	0.01	+	NA
10. 2-Ethyl-5-methylpyrazine	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-5*H*-cyclopentapyrazine	3306	32747-48-0 	34	4	0.1	+	NA
22. 6,7-Dihydro-2,3-dimethyl-5*H*-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

(*Continued on next page*)

Table 1 (*continued*)

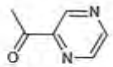
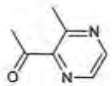
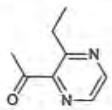
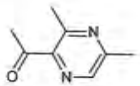
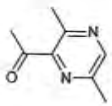
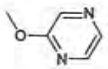
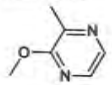
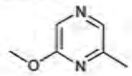
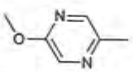
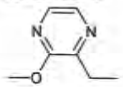
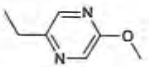
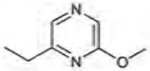
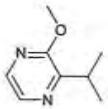
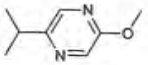
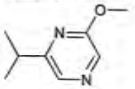
Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume (kg)	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods (kg)	Consumption ratio
				μg/day	μg/kg bw/day		
24. Acetylpyrazine	3126	22047-25-2 	923	122	2	1882	2
25. 2-Acetyl-3-methylpyrazine		23787-80-6 	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")		Annual volume in naturally occurring foods (kg)	Consumption ratio
				µg/day	µg/kg bw/day		
29. (2 or 5 or 6)-Methoxy-3-methylpyrazine	3183	2847-30-5  2882-21-5  2882-22-6 	113	15	0.2	+	NA
30. 2-Ethyl-(3 or 5 or 6)-methoxypyrazine	3280	25680-58-4  68039-50-9  67845-38-9 	11	1	0.02	+	NA
31. 2-Methoxy-(3 or 5 or 6)-isopropylpyrazine	3358	25773-40-4  56891-99-7  68039-46-3 	0.5	0.01	0.001	1	2

(*Continued on next page*)

Table 1 (*continued*)

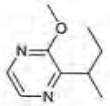
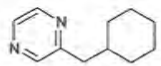
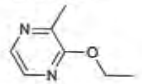
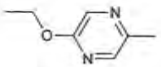
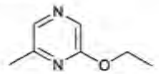
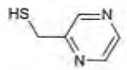
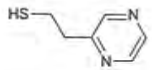
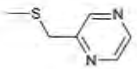
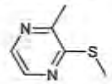
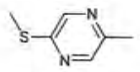
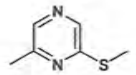
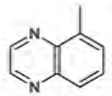
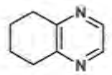
Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume (kg)	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods (kg)	Consumption ratio
				µg/day	µg/kg bw/day		
32. 2-Methoxy-3-(1-methylpropyl)-pyrazine	3433	24168-70-5 	1	0.1	0.002	+	NA
33. (Cyclohexylmethyl) 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  67845-34-5  53163-97-6 	0.05	0.01	0.0001	–	NA
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  2884-14-2  2884-13-1 	99	13	0.2	–	NA
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 Grundschober, 1987).

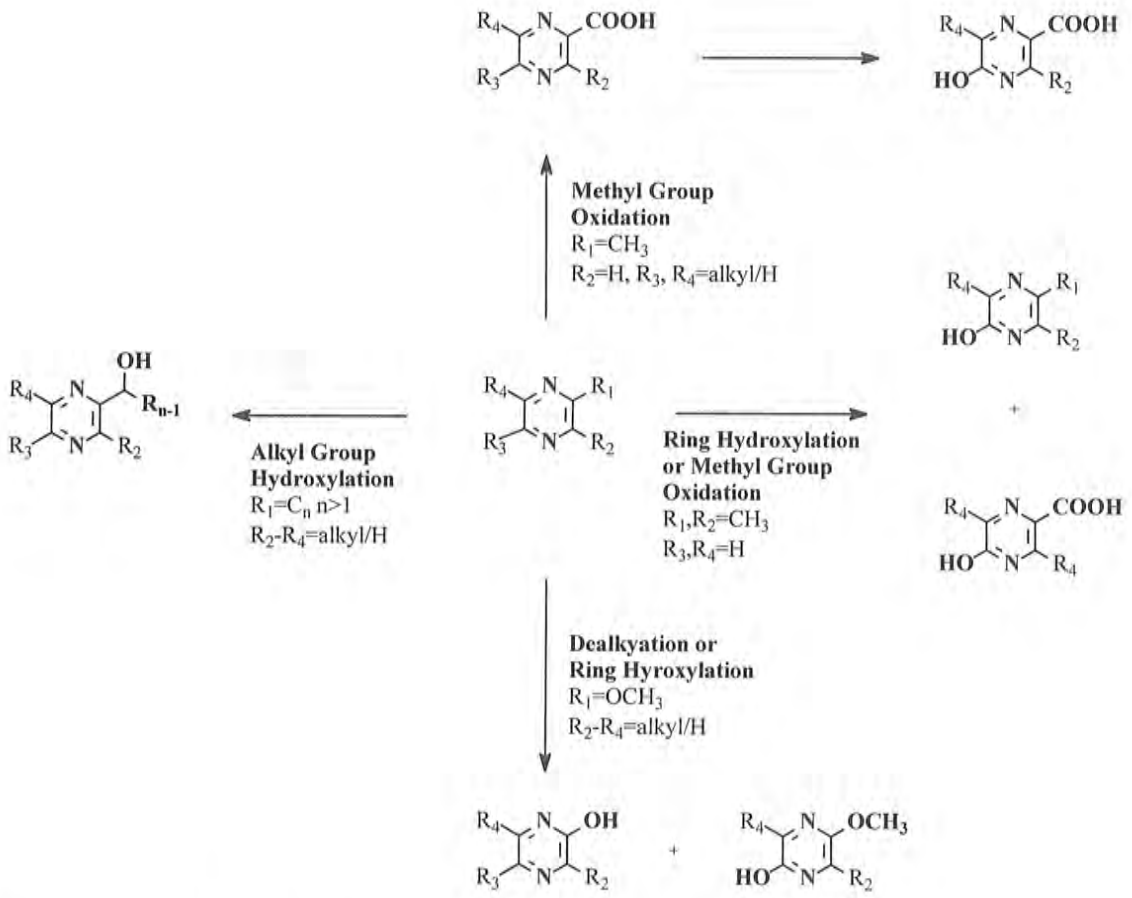
1.3. Absorption, distribution, metabolism and excretion

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 (Hawksworth 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 (Hawksworth 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 (Hawksworth 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 (Hawksworth 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 (Hawksworth 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 (Hawksworth 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; Elfarra 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 (RSO_2H) and sulfonic acid (RSO_3H); 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 LD_{50} values are available for 17 of the 41 pyrazines discussed in this review (Table 2). The rat acute oral LD_{50} 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 LD_{50} 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 LD_{50} 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-Methypyrazine	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*H*-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)	Oser (1978b)
24.	Acetylpyrazine	>3000 (Rat)	Posternak et al. (1975)	Rat, M, F	91/diet	8.2[d]	Posternak et al. (1975)
28.	Methoxypyrazine			Rat	91/diet	20	Osborne, 1981
29.	(2, 5 or 6)-Methoxy-3-methylpyrazine			Rat, M, F	90/diet	45 (M) 53 (F)	Posternak et al. (1969)
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 palability 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 intake[1] ("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 intake[1] ("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-5*H*-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 intake[1] ("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-5*H*-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-5*H*-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 LD_{50} 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 intake[1] ("eaters only") of 5-methyl-6,7-dihydro-5*H*-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 intake[1] ("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 intake[1] (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 intake[1] ("eaters only") from use of each of the three pyrazine derivatives as flavoring substances.

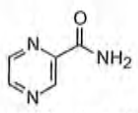
1.4.3. Carcinogenicity study with a structurally-related pyrazine

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

[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. ceravisiae* 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 ^{3}H-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 $\geqslant 70$ mg/kg body weight per day, and decreased levels of spermidine and acid phosphatase in the prostate were reported at $\geqslant 70$ 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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