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Review

The FEMA GRAS assessment of hydroxy- and alkoxy-substituted benzyl derivatives used as flavor ingredients

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Abstract

This publication is the ninth 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 and in the context of the available scientific information on the group of structurally related substances. Scientific data relevant to the safety evaluation of the use of hydroxy- and alkoxy-substituted benzyl derivatives as flavoring ingredients is evaluated. The group of hydroxy- and alkoxy-benzyl derivatives was reaffirmed as GRAS (GRASr) based, in part, on their self-limiting properties as flavoring substances in food; their rapid absorption, metabolic detoxication, and excretion in humans and other

Abbreviations: ABS, chromosomal aberration; AMS, Ames assay; *B. subtilis*, *Bacillus subtilis*; bw, body weight; CHO, Chinese hamster ovary; CoA, coenzyme A; DNA, deoxyribonucleic acid; *E. coli*, *Escherichia coli*; F, Female; FDA, United States Food and Drug Administration; FEMA, The Flavor and Extract Manufacturers Association; GRAS, Generally Recognized as Safe; GRASa, GRAS affirmed; RASr, GRAS reaffirmed; im, intramuscular; ip, intraperitoneal; iv, intravenous; LD_{50}, medianlethal dose; M, Male; MLA, mouse lymphoma cell assay; NAS, National Academy of Science; NCI, National Cancer Institute; NOAEL, no-observed-adverse effect level; NR, not reported; NTP, National Toxicology Program; ppm, parts per million; SMVCE, sperm morphology and vaginal cytology examinations; *S. typhimurium*, *Salmonella typhimurium*; SCE, sister chromatid exchanges; SLR, scientific literature review; UDS, unscheduled DNA synthesis.

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animals; their low level of flavor use; the wide margins of safety between the conservative estimates of intake and the no-observed-adverse effect levels determined from subchronic and chronic studies and the lack of significant genotoxic and mutagenic potential. This evidence of safety is supported by the fact that the intake of hydroxy- and alkoxy-substituted benzyl derivatives as natural components of traditional foods is greater than their intake as intentionally added flavoring substances.

Keywords: Hydroxybenzyl derivatives; Alkoxybenzylderivatives; Flavoring ingredients; FEMA GRAS

Contents

1. Chemical identity

This summary presents the key data relevant to the safety evaluation of 46 hydroxy- and alkoxy-substituted benzyl derivativesfor their intended use as flavoring substances (Table 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 bonded directly to a benzene ring. The ring also contains hydroxy or alkoxy substituents.

2. Exposure

2.1. Flavor use and natural occurrence

The total annual volume of the 46 flavouring agents in this group is 1,851,330 kg in the USA (NAS, 1982, 1987; Lucas et al., 1999) (see Table 1). Approximately 60% of the total annual volume in the USA is accounted for by vanillin (No. 22). Production volumes and intake values for each substance are reported in Table 1.

Twenty-nine of the 46 substances in this group of flavouring agents are naturally occurring in food. Vanillin, a major constituent of natural vanilla, is also present in strawberries and milk. Methyl salicylate, the predominant substituent of oil of wintergreen, is also found in tomatoes and grilled beef. Ethyl vanillin has been detected in raspberries and ginger, while piperonal is found in cooked chicken and pepper (Maarse et al., 1999). Quantitative natural occurrence data and consumption ratios have been reported for 13 substances in the group. Eight substances demonstrate that their consumption occurs predominantly from traditional foods (i.e. consumption ratio greater than 1) (Stofberg and Grundschober, 1987; Stofberg and Kirschman, 1985). *p*-Methoxybenzaldehyde (No. 13), methyl-*o*-methoxybenzoate (No. 15), vanillin (No. 22), methyl salicylate (No. 28), and ethyl salicylate (No. 29) are not consumed primarily from traditional foods (consumption ratio less than 1) (Stofberg and Grundschober, 1987; Stofberg and Kirschman, 1985).

3. Hydrolysis, absorption, distribution, and excretion

3.1. Hydrolysis of esters and acetals

It is expected that aromatic esters will be hydrolyzed in vivo through the catalytic activity of carboxylesterases or esterases (Heymann, 1980). A-esterases, the most important of the group, have activity in most mammalian tissue (Anders, 1989; Heymann, 1980), but predominate in the hepatocytes (Heymann, 1980). Acetals hydrolyze uncatalyzed in gastric juice and intestinal fluids to yield the corresponding aldehydes. Several in vivo experiments have reported that substituted benzyl esters and benzaldehyde acetals are hydrolyzed to the corresponding alcohols aldehydes, and carboxylic acids.

In 3- to 6-month-old male rabbits, 83% and 15% of an oral dose of 100 mg/kg bw of piperonyl acetate (No. 43) or piperonyl isobutyrate (No. 44), respectively, was hydrolyzed and excreted as either free or conjugated piperonylic acid within 72 h. In the same amount of time, less than 1% of piperonyl alcohol was excreted (Wright and Holder, 1980).

An oral dose of methyl salicylate (No. 28) equivalent to 500 mg/kg bw of salicylic acid was dissolved in 2% methylcellulose and given to male rats. The plasma levels measured within 20 min of dosing showed complete hydrolysis of methyl salicylate. A similar experiment was conducted with male dogs. Capsules, containing 320 mg methyl salicylate/kg bw, were orally administered to three fasted dogs. Blood drawn 1 h after dosing showed 95% hydrolysis of methyl salicylate to salicylic acid. In 6 humans, 79% of a 0.42 ml dose (approximately 500 mg) of methyl salicylate administered in ginger ale is hydrolyzed in the blood within the first 90 min (Davison et al., 1961).

Several experiments were conducted to study the metabolism of esters of *p*-hydroxybenzoic acid. Comparisons were made between oral (1000 mg/kg bw) and intravenous (50 mg/kg bw) administration in dogs. Methyl and ethyl esters were absorbed by the gastrointestinal tract and rapidly hydrolyzed by esterases in the liver and kidney. In the case of butyl *p*-hydroxybenzoate, 48% and 40% were recovered from the oral and intravenous administration, respectively. Liver preparations from dogs injected with 100 mg/kg bw of the methyl, ethyl, or propyl esters showed 100% hydrolysis in 3 min. In the case of the butyl ester, 100% hydrolysis occurred after 30–60 min. (Jones et al., 1956).

Benzyl acetate was rapidly hydrolyzed to benzyl alcohol (No. 1) in vivo, with peak alcohol concentration after 4 min. The absence of benzyl acetate in plasma is evidence that benzyl acetate is rapidly hydrolyzed to benzyl alcohol, which is then rapidly oxidized to benzoic acid in vivo (Yuan et al., 1995). An in vitro hydrolysis study found that benzyl phenylacetate (No. 12) was 90% hydrolyzed within 1 h and completely hydrolyzed within 2 h of incubation with a 2% pancreatin solution (Leegwater and van Straten, 1974).

Acetals of benzaldehyde are also readily hydrolyzed. Benzaldehyde propylene glycol acetal (No. 16) was 97% hydrolyzed after incubation for 5 h with simulated gastric juice and intestinal fluid in vitro compared to blank incubation (Morgaridge, 1962).

3.2. Absorption, distribution, and excretion

The hydroxy- and alkoxy-substituted benzyl derivatives have been shown to be rapidly absorbed by the gastrointestinal tract, metabolized in the liver to yield benzoic acid derivatives, and excreted primarily in the urine either unchanged or conjugated (Jones et al., 1956; Davison, 1971). To some extent metabolites participate in enterohepatic cycling leading to further metabolism by gut bacteria. Studies on the effect of dose, species, sex and mode of administration on the absorption, distribution and excretion of these substances are described in detail below.

Table 1
Identity and exposure data for hydroxy- and alkoxy-substituted benzyl derivatives used as flavor ingredients

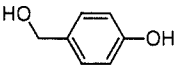
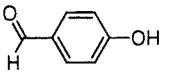
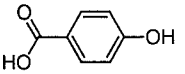
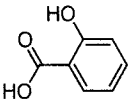
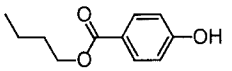
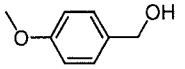
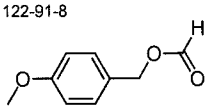
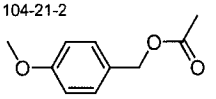
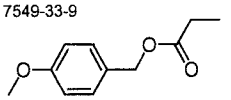
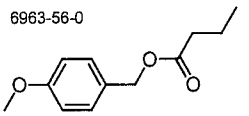
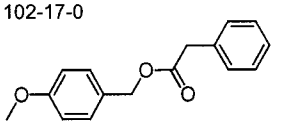
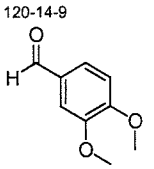
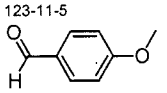
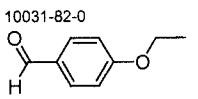
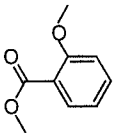
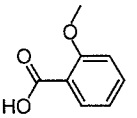
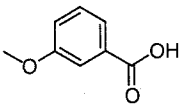
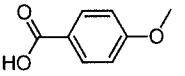
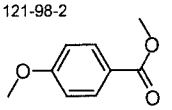
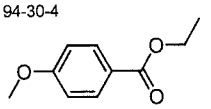
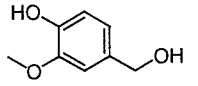
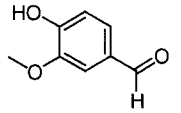
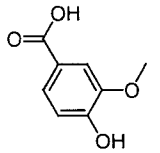
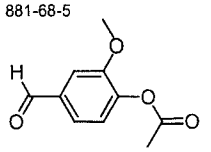
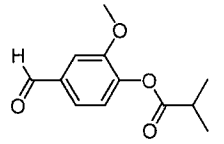
Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				µg/d	µg/kg bw/d		
1. 4-Hydroxybenzyl alcohol	3987	623-05-2 	0.4[d]	0.07	0.001	+	NA
2. 4-Hydroxybenzaldehyde	3984	123-08-0 	450[d]	79	1	3567	8
3. 4-Hydroxybenzoic acid	3986	99-96-7 	131[d]	23	0.4	23,185	177
4. 2-Hydroxybenzoic acid	3985	69-72-7 	0.2[d]	0.04	0.0006	20,021	100,105
5. Butyl *p*-hydroxybenzoate	2203	94-26-8 	0.2[e]	0.04	0.0006	–	NA
6. Anisyl alcohol	2099	105-13-5 	441	58	1	+	NA
7. Anisyl formate	2101	122-91-8 	182	24	0.4	–	NA
8. Anisyl acetate	2098	104-21-2 	2250	296	5	+	NA
9. Anisyl propionate	2102	7549-33-9 	37	5	0.1	–	NA
10. Anisyl butyrate	2100	6963-56-0 	1	0.1	0.002	–	NA
11. Anisyl phenylacetate	3740	102-17-0 	0.5[f]	0.09	0.001	–	NA
12. Veratraldehyde	3109	120-14-9 	414	55	1	+	NA
13. *p*-Methoxybenzaldehyde	2670	123-11-5 	4391	578	10	77	0.02
14. *p*-Ethoxybenzaldehyde	2413	10031-82-0 	0.1	0.01	0.0002	+	NA

Table 1 (*continued*)

Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				μg/d	μg/kg bw/d		
15. Methyl *o*-methoxybenzoate	2717	606-45-1 	64	8	0.1	25	0.4
16. 2-Methoxybenzoic acid	3943	579-75-9 	0.1[d]	0.02	0.0003	+	NA
17. 3-Methoxybenzoic acid	3944	586-38-9 	0.1[d]	0.02	0.0003	+	NA
18. 4-Methoxybenzoic acid	3945	100-09-4 	1[d]	0.2	0.003	116	116
19. Methyl anisate	2679	121-98-2 	0.05	0.01	0.0001	33	660
20. Ethyl *p*-anisate	2420	94-30-4 	16	2	0.04	+	NA
21. Vanillyl alcohol	3737	498-00-0 	46	6	0.1	+	NA
22. Vanillin	3107	121-33-5 	1,140,909	150,278	2505	20,075	0.02
23. 4-Hydroxy-3-methoxy benzoic acid	3988	121-34-6 	200[d]	35	0.6	101,140	506
24. Vanillin acetate	3108	881-68-5 	6	0.7	0.01	+	NA
25. Vanillin isobutyrate	3754	20665-85-4 	0.3	0.04	0.0007	–	NA

(*continued on next page*)

Table 1 (*continued*)

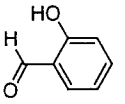
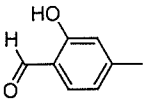
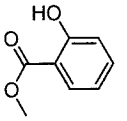
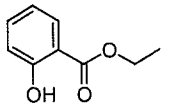
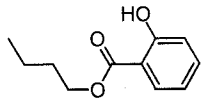
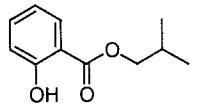
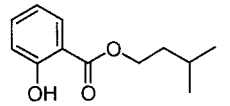
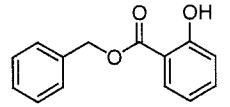
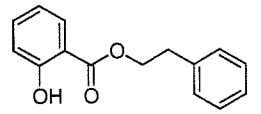
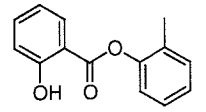
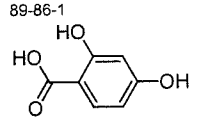
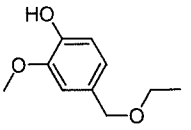
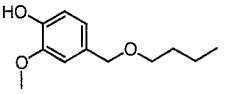
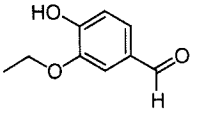
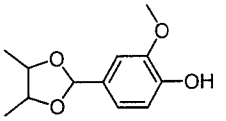
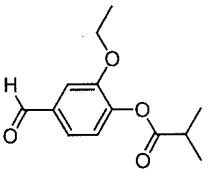
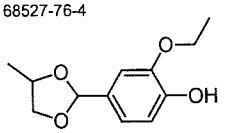
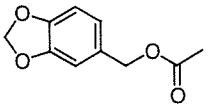
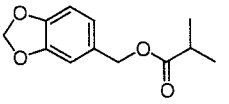
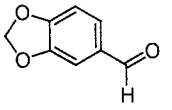
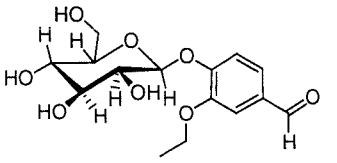
Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				µg/d	µg/kg bw/d		
26. Salicaldehyde	3004	90-02-8 	123	16	0.3	66,470	540
27. 2-Hydroxy-4-methylbenzaldehyde	3697	698-27-1 	2	0.3	0.004	+	NA
28. Methyl salicylate	2745	119-36-8 	337,273	44,425	740	2493	0.01
29. Ethyl salicylate	2458	118-61-6 	13,046	1718	29	9	0.0007
30. Butyl salicylate	3650	2052-14-4 	0.05	0.006	0.0001	+	NA
31. Isobutyl salicylate	2213	87-19-4 	43	6	0.1	–	NA
32. Isoamyl salicylate	2084	87-20-7 	55	7	0.1	14,286	260
33. Benzyl salicylate	2151	118-58-1 	223	29	0.5	+	NA
34. Phenethyl salicylate	2868	87-22-9 	32	4	0.07	–	NA
35. *o*-Tolyl salicylate	3734	617-01-6 	227[d]	40	0.7	–	NA
36. 2,4-Dihydroxybenzoic acid	3798	89-86-1 	45[d]	8	0.1	+	NA

Table 1 (*continued*)

Flavoring ingredient	FEMA no.	CAS no. and structure	Most recent annual volume, kg[a]	Daily per capita intake ("eaters only")		Annual volume in naturally occurring foods, kg[b]	Consumption ratio[c]
				µg/d	µg/kg bw/d		
37. Vanillyl ethyl ether	3815	13184-86-6 	165[d]	29	0.5	–	NA
38. Vanillyl butyl ether	3796	82654-98-6 	1	0.1	0.002	–	NA
39. Ethyl vanillin	2464	121-32-4 	325,455	42,868	714	–	NA
40. Vanillin *erythro*- and *threo*-butan-2,3-diol acetal	4023	63253-24-7 	25[d]	4	0.07	–	NA
41. Ethyl vanillin isobutyrate	3837	188417-26-7 	9[d]	2	0.03	–	NA
42. Ethyl vanillin propylene glycol acetal	3838	68527-76-4 	275[d]	48	0.8	–	NA
43. Piperonyl acetate	2912	326-61-4 	82	11	0.2	+	NA
44. Piperonyl isobutyrate	2913	5461-08-5 	26	3	0.06	–	NA
45. Piperonal	2911	120-57-0 	24,455	3221	54	+	NA
46. Ethyl vanillin β-D-glucopyranoside	3801	122397-96-0 	227[d]	40	0.7	–	NA

(*continued on next page*)

Table 1 (*continued*)

[a] Intake (μg/person/day) 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; where correction factor = 0.6 for NAS surveys and 0.8 for the Lucas et al. USA survey representing the assumption that only 60% and 80% of the annual flavor volume, respectively, was reported in the poundage surveys Lucas et al. (1999); NAS (1982, 1987). Intake (μg/kg bw/d) calculated as follows: [(μg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[b] Quantitative data for the United States reported by Stofberg and Grundschober (1987).

[c] The consumption ratio is calculated as follows: (annual consumption via food, kg)/(most recent reported volume as a flavoring substance, kg); NA = data not available.

[d] The volume cited is the anticipated annual volume, which was the maximum amount of flavor estimated to be used annually by the manufacturer at the time the material was proposed for flavor use.

[e] NAS (1982).

[f] NAS (1987).

In rabbits, 96% of a single oral dose of 400 mg/kg bw 4-hydroxybenzaldehyde (No. 2) is excreted in the urine within 24 h as 4-hydroxybenzoic acid and its glycine, glucuronic acid and sulfate conjugates (Bray et al., 1952).

In a similar study, groups of 4–8 rabbits were given doses of 100, 250, 500, 1000, or 1500 mg 4-hydroxybenzoic acid/kg bw (No. 3) by gavage every three to seven days. Urine was collected continuously and analyzed for metabolites. Total urinary recovery of the test material was in the range from 84% to 104% with ether soluble acids comprising 64% to 75% of the total. Glucuronic acid and sulfate conjugates were also detected in the urine at 10% to 35% and 4% to 7%, respectively. The levels for all the metabolites returned to background levels within 24 h after dosing (Bray et al., 1947). In a corresponding study, approximately 94% of a single oral dose of 250 or 500 mg 2-hydroxybenzoic acid/kg bw (No. 4) given to two groups of four rabbits was excreted unchanged or as the glucuronic acid and sulfate conjugates (Bray et al., 1948).

In female albino rats, approximately 6% of a 52.4 mg dose of 2,4-dihydroxybenzaldehyde (No. 36) given by intraperitoneal injection was excreted in the urine as the corresponding hippurate within 24 h (Teuchy et al., 1971). In humans being treated for rheumatic fever, three patients were given daily oral doses of 5330–6000 mg of 2,4-dihydroxybenzoic acid in 1000 mg doses every 3 h for 2–16 days. Average daily urinary excretion rates were 42.7–75.8%. Average daily excretion of sulfate conjugate per patient was essentially constant during the study, but average daily excretion of glucuronic acid conjugate increased 4–6-fold over the 16 day period (Clarke et al., 1958). In an investigation of the presence of dihydroxybenzoic acid isomers in the urine of humans (15), only 3,5-dihydroxybenzoic acid was detected (Williams, 1965).

Groups of three or more fasted dogs were given 1000 mg butyl *p*-hydroxybenzoate (No. 5)/kg bw orally, or 50 mg/kg bw by intravenous (iv) injection. Blood and urine samples were collected at fixed intervals until the levels returned to background within 48 h. Recovery of the total test material, almost entirely as the *p*-hydroxybenzoic acid conjugate of glucuronic acid, after the oral and intravenous doses was 48% and 40%, respectively. Most of the material was excreted between 6 and 30 h after dosing. Although the relatively low rate of recovery seen in both dosing methods was attributed to incomplete hydrolysis of the ester in the body, in vitro incubation of the butyl ester with freshly prepared liver homogenate showed complete hydrolysis within 30–60 min. Studies conducted with other related benzoate esters, such as methyl and ethyl *p*-hydroxybenzoate, showed significantly higher rates of material recovery (Jones et al., 1956). This suggests that an increase in the homologous series of alkyl esters may result in the activation of other metabolic and excretion pathways. Overall, the authors concluded that butyl *p*-hydroxybenzoate and other alkyl esters are readily absorbed, metabolized, and excreted by the body.

A group of 10 rabbits were each fed 200 mg veratraldehyde (No. 12; 3,4-dimethoxybenzaldehyde) via stomach tube and urine was collected over the next 24 h. Approximately 70% of the material was recovered in the urine as free corresponding acid (~28%) and its glucuronic acid (~38%) or sulfate (3–7%) conjugate (Sammons and Williams, 1941).

Because of its prevalence and importance as a flavouring agent, vanillin (No. 22; 4-hydroxy-3-methoxybenzaldehyde) has been the subject of numerous metabolism studies. In male albino rats, 100 mg vanillin/kg bw in a solution of propylene glycol and water was administered by stomach tube. Urine and feces were collected separately for 24-h periods, and bile samples were collected by cannulation of the common bile duct. Only trace amounts of benzoic acid derivatives remained in the urine after the first 24 h and none after 48 h. Ninety four percent (94%) of the dose was accounted for in the urine as free and conjugated forms of vanillic acid and vanillyl alcohol. Vanillin and its primary reduction and oxidation metabolites were also excreted in appreciable amounts in the bile. Bile collected for 5 h after two rats were given 100 and 300 mg/kg bw oral

doses of vanillin contained glucuronide conjugates of vanillin (6%), vanillyl alcohol (8%), and vanillic acid (9%) (Strand and Scheline, 1975).

In Sprague-Dawley albino rats, 60% of a 100 mg/kg bw dose of vanillin in 0.9% NaCl given by intraperitoneal (ip) injection was recovered in the 24-h urine. Urinary metabolites included unconjugated vanillic acid, the sulfate and glucuronic acid conjugates of vanillic acid, conjugates of vanillyl alcohol, vanillin, and catechol. The presence of the urinary glycine conjugate of vanillic acid was not reported in this study (Wong and Sourkes, 1966).

Three (3) rabbits fed 1000 mg vanillin/kg bw by gavage excreted, on average, 83% of the dose in the urine. Sixty nine percent (69%) of the dose was recovered as free and conjugated vanillic acid, and 14% as conjugated vanillin (Sammons and Williams, 1941). A 100 mg dose of vanillin dissolved in water was given to an adult human and the urine collected for 24 h. Examination revealed an increase in the vanillic acid output in the urine from a background level of 0.3 mg/24 h to 96 mg/24 h. The observed increase accounted for approximately 94% of the vanillin dose (Dirscherl and Wirtzfeld, 1964).

A single dose of 400 mg salicylaldehyde (No. 26)/kg bw was administered to a fasted rabbit. Approximately 75% of the dose was excreted in the urine collected over 24 h. Urine analysis revealed mainly ether soluble acids, with 27% and 3% accounted for as glucuronic acid and sulfate conjugates of vanillic acid, respectively (Bray et al., 1952).

In male rats, 94% of a 150 mg/kg bw dose of piperonal (No. 45) in propylene glycol administered by gavage was accounted for in the urine within 24 h. No unchanged compound was excreted and no metabolites were detected in the urine more than 48 h after the dose was administered (Klungsoyr and Scheline, 1984). The metabolism of corresponding esters piperonyl acetate (No. 43) and piperonyl isobutyrate (No. 44) were also studied. In male rabbits, 70.5% of a 100 mg/kg bw dose of piperonyl acetate and 11.4% of a 100 mg/kg bw dose of piperonyl isobutyrate, both administered by gavage, were recovered in the 72-h urine (Wright and Holder, 1980).

Based on these studies, it is anticipated that the hydroxy- and alkoxy-substituted benzyl derivatives are rapidly absorbed, metabolized, and excreted in the urine mainly as sulfate and glucuronic acid conjugates of the corresponding hydroxybenzoic acid derivatives.

3.3. Metabolism

3.3.1. Metabolism of alcohol, aldehydes, and other derivatives

In general, hydroxy- and alkoxy-derivatives of benzaldehyde and benzyl alcohol are oxidized to the corresponding benzoic acid derivatives and, to a lesser extent, reduced to the corresponding benzyl alcohol derivatives. Resulting hydroxy- and alkoxy-benzoic acid derivatives form sulfate, glucuronic acid, or glycine conjugates depending mainly on ring substitution. Hydroxy- and methoxy-substituted benzoic acid derivatives (e.g., vanillic acid) tend to form sulfate or glucuronic acid conjugates while methylenedioxy-substituted benzoic acid derivatives (e.g., piperonylic acid) form glycine conjugates. To a minor extent, benzoic acid hydroxy- and alkoxy-derivatives undergo decarboxylation and O-demethylation. Protocatechuic acid is a key intermediate formed via the O-demethylation of benzoic acid (Wong and Sourkes, 1966; Strand and Scheline, 1975). Benzyl alcohol derivatives also, may be reduced in gut micro flora to toluene derivatives, especially if a free *p*-hydroxyl group is present (Strand and Scheline, 1975) (see Fig. 1).

In a study of the degradation of some methoxylated aromatic compounds by *Actinomyces aureus* A-94 (Tsai et al., 1965), anisyl alcohol (No. 6) was oxidized to corresponding anisic acid then demethylated and hydroxylated to yield protocatechuic acid (3,4-dihydroxybenzoic acid). Additional enzymatic action converted protocatechuic acid to succinic acid via β-carboxymuconic acid and β-oxoadipic acid. In the final stage, succinic acid entered the tricarboxylic cycle (TCA).

In an in vitro study in animal cell preparations, anisyl alcohol was incubated with rat cecal extract. Analysis after approximately 46 h showed the presence of anisic acid. O-demethylation was not observed (Scheline, 1972).

In the investigation of the metabolism of various aromatic aldehydes and alcohols by rat intestinal microflora, the major metabolites were products of reduction. Mediated-metabolic transformations included reduction, dehydroxylation, O-demethylation, and decarboxylation leading to a variety of benzyl alcohol, benzoic acid, and toluene derivatives (Scheline, 1972). Incubation of vanillyl alcohol (No. 21) with rat cecal extract showed the presence of vanillic acid, and the toluene derivatives, 4-methylguaiacol and 4-methylcatechol, forming from complete reduction of the alcohol functional group (Scheline, 1972). In an in vivo study conducted in male rats, vanillyl alcohol was administered by gavage in doses of 100 or 300 mg/kg bw. Analysis of the urinary metabolites collected over the first 24-h period showed the presence of vanillic acid and trace amounts of vanillyl alcohol and the glycine conjugate of vanillic acid. Also identified in smaller quantities were conjugated fractions of vanillin, guaiacol, catechol, 4-methylguaiacol, and 4-methylcatechol. The presence of catechol and 4-methylcatechol indicate that decarboxylation and complete reduction of the alcohol function, respectively, occur in vivo (Strand and Scheline, 1975).

A 2000 mg/kg bw oral dose of veratraldehyde (No. 12; 3,4-dimethyoxybenzaldehyde) was administered to

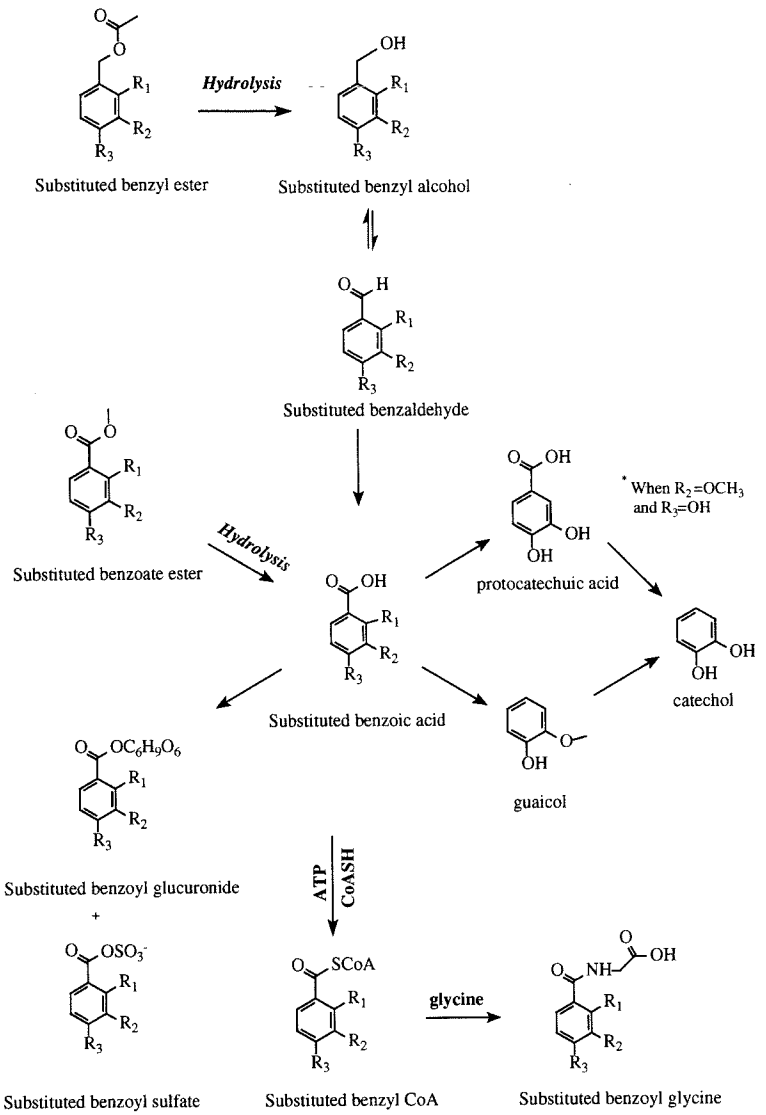


Fig. 1. Metabolism of hydroxy- and alkoxy-benzene derivatives.

rabbits by gavage and urine was collected for 24 h. Approximately 70% of the aldehyde was accounted for in urine mainly as the corresponding acid, veratric acid (28%) and its glucuronic acid conjugate (38%). To a small extent, veratric acid was decarboxylated and O-demethylated to yield catechol (Sammons and Williams, 1946). Presumably, veratric acid enters enterohepatic circulation where gut micro flora decarboxylate the acid to yield catechol (*o*-hydroxyphenol). The observation that catechol, is formed when veratraldehyde is incubated with rat cecal extract is evidence for this decarboxylation pathway in gut bacteria (Scheline, 1972).

The analysis of metabolites produced 46 h after the incubation of anisaldehyde (No.13; *p*-methoxybenzaldehyde) with rat cecal preparations revealed the presence of anisic acid and anisyl alcohol. These results show that anisaldehyde undergoes oxidation and reduction in cecal preparations (Scheline, 1972). In rabbits, approximately 75% of an oral dose of 2000 mg/kg bw of anisaldehyde is excreted as the glucuronic acid conjugate of *p*-methoxybenzoic acid (anisic acid) within 24 h (Sammons and Williams, 1946).

In male rats (4-6/group), a single oral dose of 100 or 300 mg/kg bw of vanillin (No. 22) was metabolized and excreted in the urine as vanillin, vanillic acid, and vanillyl alcohol within the first 24 h. Minor amounts of O-demethylated, decarboxylated and further reduced metabolites were also identified. These included protocatechuic acid (3,4-dihydroxybenzoic acid, product of O-demethylation), guaiacol (*o*-methoxyphenol, product of decarboxylation), vanilloylglycine, catechol (*o*-hydroxyphenol), 4-methylguaiacol (product of alcohol functional group reduction), and 4-methylcatechol (product of reduction and ring hydroxylation). Only traces of vanillic acid derivatives were detected in the urine collected between the 24 and 48-h period, and no

metabolites were detected in urine collected between 48 and 96 h. In a similar experiment in rats, greater than 94% of a single oral dose of 100 mg/kg bw of vanillin is excreted in the urine within 48 h. Between 65% and 70% of the urinary metabolites were oxidation products (Strand and Scheline, 1975).

Most of a 100 mg/kg bw dose of vanillin administered by ip injection to rabbits was excreted in the urine 24 h after dosing. Approximately 69% of vanillin was oxidized to vanillic acid and 10% was reduced to vanillyl alcohol. In addition, over 10% was excreted as the glucuronic acid conjugate of vanillin (Sammons and Williams, 1941).

In rats, less than 6% of a dose of 52 mg of 2,4-dihydroxybenzoic acid given by ip injection is excreted as the corresponding hippurate (Teuchy et al., 1971). In humans given daily oral doses of up to 6000 mg of 2,4-dihydroxybenzoic acid for up to 16 days, the major urinary metabolites were the glucuronic acid and sulfate conjugates (Clarke et al., 1958).

In albino male Wistar rats, 94% of a 150 mg/kg bw oral dose of piperonal (No. 45) or 90% of a 150 mg/kg bw dose of piperonyl alcohol dissolved in propylene glycol was excreted in the urine within 24 h. The primary urinary metabolites for either substance were piperonylic acid (17–20%) or the glycine conjugate of piperonylic acid (71–72%). No unchanged compound was excreted and no metabolites were detected 48 h after dosing. Less than 0.7% of the dose was accounted for by O-demethylenation to yield protocatechuyl alcohol, protocatechualdehyde, and protocatechuic acid (Klungsoyr and Scheline, 1984).

In male Swiss-Webster mice, 87–93% of an 0.75 mg/kg bw oral dose of either radiolabelled (methylene-^{14}C) piperonyl alcohol, piperonal, or piperonylic acid administered in DMSO (50 μl followed by a 100 μl wash) was accounted for in the urine within 48 h. The majority was eliminated within the first 12 h. Less than 10% was excreted in the feces. In all cases, the major metabolite was the glycine conjugate of piperonylic acid. Minor amounts of unchanged piperonylic acid were also present (Kamienski and Casida, 1970).

3.3.2. Summary of metabolism

It is expected that esters of hydroxy- and alkoxy-substituted benzyl derivatives are hydrolyzed to the corresponding benzyl alcohol or benzoic acid derivatives, while the acetals are hydrolyzed to the parent benzaldehyde derivative. Thus formed, the alcohols and aldehydes are oxidized mainly to benzoic acid derivatives that are either excreted unchanged or form sulfate, glycine, or glucuronic acid conjugates. To some extent, glucuronic acid conjugates may pass into the bile and enter enterohepatic circulation where these metabolites are hydrolyzed or subjected to the reduction reactions of gut bacteria. Minor amounts of hydroxy- and alkoxy-benzoic acid derivations have been reported to undergo reductive decarboxylation in the gut. Other minor metabolic detoxication pathways include O-demethylation, and ring hydroxylation.

4. Toxicological studies

4.1. Acute toxicity

Oral LD_{50} values for 36 of the 46 benzyl derivatives have been reported, and range from 520 mg/kg bw to 13,200 mg/kg bw in male and female rats, guinea pigs, mice, or rabbits (Deichmann and Kitzmiller, 1940; Draize et al., 1948; Sokol, 1952;Giroux et al., 1954; Anonymous, 1956; Davison et al., 1961; Doull et al., 1962; Jenner et al., 1964; Taylor et al., 1964; Hagan et al., 1965; Fogleman and Margolin, 1970; Weir and Wong, 1971; Moreno, 1973, 1974, 1976, 1977, 1982; Sado, 1973; Wohl, 1974; Levenstein, 1975; Grady et al., 1976; BASF, 1981; Givaudan, 1982; Mondino, 1982; Peano and Berruto, 1982; Mallory et al., 1983; Sterner and Chibanguza, 1983; NTP, 1984a,b; Ohsumi et al., 1984; Ohta et al., 1984; Reagan and Becci, 1984; Inouye et al., 1988; Buch, 1989; Hasegawa et al., 1989; Cerven, 1990; Dow, 1992; Dufour, 1994; Sanders and Crowther, 1997). The majority of the compounds have an LD_{50} value greater than 1000 mg/kg bw indicating very low oral toxicity (see Table 2).

4.2. Short-term studies of toxicity

Forty-five (45) short-term studies have been performed for 8 of the substances in this group. The vast majority of these studies were conducted for butyl *p*-hydoxybenzoate (No. 5), commonly recognized as butyl paraben, vanillin (No. 22), methyl salicylate (No. 28), ethyl vanillin (No. 39), and piperonal (No. 45). They are summarized below and in Table 2.

4.2.1. Mice

4.2.1.1. Butyl-p-hydroxybenzoate (No. 5). For a period of 6 weeks, 5 groups of 10 male and 10 female 8-week-old ICR/jcl mice were maintained on diets containing pelletized butyl-*p*-hydroxybenzoate at levels calculated to provide an average daily intake of 900, 1875, 3750, 7500, or 15,000 mg/kg bw per. Twenty male and 20 female mice of the same age and strain were used as control and fed the basal diet. Survival, body-weight gain measurement, and histopathological examination were performed. All of the animals in the 7500 and 15,000 mg/kg bw per day groups died within the first 2 weeks of treatment. The body-weight gain in the 1875 and 3750 mg/kg bw per day groups was less than 10% of the control group, whereas the animals in the lowest-dose group showed similar weight gain to the control. Further, histological

Table 2
Acute and short-term toxicity studies for hydroxy- and alkoxy-substituted benzyl derivatives used as flavor ingredients

	Flavoring ingredient	Oral acute studies		Short-term studies			
		Oral LD_{50} mg/kg bw (Species)	Reference	Species, Sex[a]	Time (days)/route	NOAEL (mg/kg bw)	Reference
2.	4-Hydroxybenzaldehyde	3980 (Rat)	Dow (1992)				
2.	4-Hydroxybenzaldehyde	500–1000 (Mouse)	Doull et al. (1962)				
3.	4-Hydroxybenzoic acid	2200 (Mouse)	Sokol (1952)				
3.	4-Hydroxybenzoic acid	210 (Mouse)	Matthews et al. (1956)				
4.	2-Hydroxybenzoic acid	908 (Mouse)	Sado (1973)				
4.	2-Hydroxybenzoic acid	1050 (Rat)	Hasegawa et al. (1989)				
5.	Butyl-*p*-hydroxybenzoate	13,200 (Mouse)	Sado (1973)	Mouse, M, F	42/Oral	900	Inai et al. (1985)
5.	Butyl-*p*-hydroxybenzoate	>5000 (Mouse)	Sokol (1952)	Rat	91-105/ Gavage	50[b]	Ikeda and Yokoi (1950)
5.	Butyl-*p*-hydroxybenzoate			Mouse, M, F	714/Oral	900[b]	Inai et al. (1985)
5.	Butyl-*p*-hydroxybenzoate			Rat, M, F	84/Oral	2000	Matthews et al. (1956)
6.	Anisyl alcohol	1780 (Mouse)	Draize et al. (1948)				
6.	Anisyl alcohol	1340 (Rat)	Draize et al. (1948)				
7.	Anisyl formate	1770 (Rat)	Levenstein (1975)				
8.	Anisyl acetate	2250 (Rat)	Weir and Wong (1971)				
9.	Anisyl propionate	3330 (Rat)	Wohl (1974)				
10.	Anisyl butyrate	3400 (Rat)	Moreno (1976)				
11.	Anisyl phenylacetate	4641(F) (Rat) 5417(M) (Rat)	Reagan and Becci (1984)				
11.	Anisyl phenylacetate	>5000 (Rat)	Moreno (1977)				
12.	Veratraldehyde	2000 (Rat)	Moreno (1974)				
13.	*p*-Methoxybenzaldehyde	3210 (Rat)	BASF (1981)	Rat, M, F	84/Oral	7.3[c,b]	Trubeck (1958)
13.	*p*-Methoxybenzaldehyde	1510 (Rat)	Jenner et al. (1964)	Rat, M, F	189-196/Oral	50[b]	Hagan et al. (1967)
13.	*p*-Methoxybenzaldehyde	1260 (Guinea pig)	Jenner et al. (1964)	Rat, M, F	105	500[b]	Hagan et al. (1967)
13.	*p*-Methoxybenzaldehyde	1510 (Rat)	Taylor et al. (1964)				
14.	*p*-Ethoxybenzaldehyde	2100 (Rat)	Moreno (1977)				
15.	Methyl-*o*-methoxybenzoate	3800 (Rat)	Moreno (1982)	Rat, M, F	14/Oral	94[b]	Van Miller and Weaver (1987)
19.	Methyl anisate	>5000 (Rat)	Levenstein (1975)				
20.	Ethyl *p*-anisate	2240 (Rat)	Levenstein (1975)				
21.	Vanillyl alcohol	>640 (Mouse)	Fujii et al. (1970)				
22.	Vanillin	1000 (Mouse)	Inouye et al. (1988)	Rat, M, F	189-196/Oral	50[b]	Hagan et al. (1967)
22.	Vanillin	2600 (Rabbit)	Deichmann and Kitzmiller (1940)	Rat, M, F	365/Oral	2500[b]	Hagan et al. (1967)
22.	Vanillin	1580 (Rat)	Taylor et al. (1964)	Rat, M, F	112/Oral	500[b]	Hagan et al. (1967)
22.	Vanillin	1580 (Rat)	Jenner et al. (1964)	Rat, M, F	730/Oral	1000[b]	Hagan et al. (1967)
22.	Vanillin	1400 (Guinea pig)	Jenner et al. (1964)				
22.	Vanillin			Rat	70[d]/Oral	64[b]	Deichmann and Kitzmiller (1940)
22.	Vanillin			Rat	126/Oral	20[b]	Deichmann and Kitzmiller (1940)
22.	Vanillin			Rabbit	56 or 126/ Oral	240[b]	Deichmann and Kitzmiller (1940)
22.	Vanillin			Rat	98/ Gavage	300[b,e]	Deichmann and Kitzmiller (1940)
22.	Vanillin			Rat, M, F	14 or 61/Oral	83[b]	Deichmann and Kitzmiller (1940)
23.	4-Hydroxy-3-methoxybenzoic acid	>2691 (Mouse)	Ohta et al. (1984)				
23.	4-Hydroxy-3-methoxybenzoic acid	5020 (Rat)	Anonymous (1956)				

25.	Vanillin isobutyrate	4755 (Rat)	Mallory et al. (1983)				
26.	Salicylaldehyde	520 (Rat)	Moreno (1977)				
27.	2-Hydroxy-4-methylbenzaldehyde	1520 (Rat)	Mondino (1982)				
27.	2-Hydroxy-4-methylbenzaldehyde	1520 (Rat)	Peano and Berruto (1982)				
28.	Methyl salicylate	1390 (Mouse)	Ohsumi et al. (1984)	Rat, M, F	Up to 71/Oral	<1000[f]	Webb and Hansen (1963)
28.	Methyl salicylate	1250 (Rat)	Giroux et al. (1954)	Rat, M	42/Oral	<300[g]	Abbott and Harrisson (1978)
28.	Methyl salicylate	2642 (F) (Rat) 3,049(M) (Rat)	Givaudan (1982)	Rat, M, F	77/Oral	450	Abbott and Harrisson (1978)
28.	Methyl salicylate	887 (Rat)	Jenner et al. (1964)	Rat, M	84/Oral	300	Abbott and Harrisson (1978)
28.	Methyl salicylate	1110 (Mouse)	Davison et al. (1961)	Rat, M, F	119/Oral	500[b]	Webb and Hansen (1963)
28.	Methyl salicylate	1060 (Guinea pig)	Jenner et al. (1964)	Dog	59/Oral	250	Webb and Hansen (1963)
28.	Methyl salicylate	1440 (Mouse)	NTP (1984a, 1984b)	Rat, M, F	77/Oral	<1000[f]	Abbott and Harrisson (1978)
28.	Methyl salicylate			Dog M, F	225[h]/Oral	<150	Abbott and Harrisson (1978)
28.	Methyl salicylate			Dog, M, F	180[i]/Oral	167[b]	Abbott and Harrisson (1978)
28.	Methyl salicylate			Rat, M, F	77/Oral	180	Abbott and Harrisson (1978)
28.	Methyl salicylate			Rat	70/Oral	<560	Harrison et al. (1963)
28.	Methyl salicylate			Rat, M, F	730/Oral	50	Webb and Hansen (1963)
28.	Methyl salicylate			Dog, M, F	730/Oral	50	Webb and Hansen (1963)
28.	Methyl salicylate			Rat, M, F	730/Oral	105[b]	Packman et al. (1961)
28.	Methyl salicylate			Mice, M, F	126/ Gavage	250	NTP (1984a, 1984b)
29.	Ethyl salicylate	1320 (Rat)	Moreno (1976)				
30.	Butyl salicylate	1836 (Rat)	Levenstein (1975)				
31.	Isobutyl salicylate	1560 (Rat)	Moreno (1973)				
32.	Isoamyl salicylate	4100 (Rat)	Moreno (1982)	Rat	98/Oral	250[j]	Drake et al. (1975)
32.	Isoamyl salicylate	>5000 (Rat)	Givaudan (1982)	Rat, M, F	91/Oral	2.5	Drake et al. (1975)
33.	Benzyl salicylate	2227 (Rat)	Fogleman and Margolin (1970)				
34.	Phenethyl salicylate	>5000 (Rat)	Moreno (1973)				
35.	*o*-Tolyl salicylate	1.81 ml/kg (Rat)	Sterner and Chibanguza (1983)				
36.	2,4-Dihydroxybenzoic acid	>800 (Rat)	Grady et al. (1976)				
37.	Vanillyl ethyl ether	>2000 (Rat)	Dufour (1994)				
38.	Vanillyl butyl ether	4734 (F) (Rat) 5104 (M) (Rat)	Buch (1989)				
39.	Ethyl vanillin	>2000 (Rat)	Jenner et al. (1964)	Rat, M	365/Oral	2500[b]	Hagan et al. (1967)
39.	Ethyl vanillin	2000 (Rabbit)	Deichmann and Kitzmiller (1940)	Rat, M, F	730/Oral	1000[b]	Hagan et al. (1967)
39.	Ethyl vanillin			Rat, M, F	91/Oral	500	Hooks et al. (1992)
39.	Ethyl vanillin			Rabbit	56 or 126/Oral	240[b]	Deichmann and Kitzmiller (1940)
39.	Ethyl vanillin			Rat	70[d]/Oral	64[b]	Deichmann and Kitzmiller (1940)
39.	Ethyl vanillin			Rat	126[d]/Oral	20[b]	Deichmann and Kitzmiller (1940)
39.	Ethyl vanillin			Rat	98/Gavage	300[b]	Deichmann and Kitzmiller (1940)
39.	Ethyl vanillin			Rabbit	15-49/Oral	41[b]	Deichmann and Kitzmiller (1940)
41.	Ethyl vanillin isobutyrate	>2000 (Rat)	Sanders and Crowther (1997)				
43.	Piperonyl acetate	2100 (Rat)	Moreno (1973)				
45.	Piperonal	2700 (Rat)	Jenner et al. (1964)	Rat, M, F	196/Oral	50[b]	Hagan et al. (1967)
45.	Piperonal	2700 (Rat)	Taylor et al. (1964)	Rat, M, F	112/Oral	500[b]	Hagan et al. (1967)
45.	Piperonal	2700 (Rat)	Hagan et al. (1965)	Rat, M, F	105/Oral	500[b]	Hagan et al. (1967)
45.	Piperonal			Rat, M, F	189-196/Oral	50[b]	Hagan et al. (1967)
45.	Piperonal			Rat, M, F	84/Oral	16[c,b]	Trubeck (1958)
45.	Piperonal			Rat, M, F	548-730/Oral	250[b]	Bar and Griepentrog (1967)
46.	Ethyl vanillin β-D-glucopyranoside	>5000 (Rat)	Cerven (1990)				

Table 2 (*continued*)

[a] M = Male; F = Female. If not listed, sex was not specified in the report.

[b] This study was performed at either a single dose level or multiple dose levels that produced no adverse effects. Therefore, this dose level is not a true NOAEL, but is the highest dose tested that produced no adverse effects. The actual NOAEL would be higher.

[c] Rats were fed a test mixture containing 123 ppm of Eugenol, 10 ppm of *p*-Methoxybenzaldehyde, and 22 ppm of Piperonal.

[d] Low dose for 126 days. 70 days for high dose, with an 8 week recovery period for half of these rats.

[e] Compound was administered two times per week.

[f] This study was performed at a single dose that produced adverse effects. The actual NOAEL would be lower.

[g] 0.6% pair-fed rats showed adverse effects, however those fed ad libitum did not.

[h] Two animals from the 150 mg/kg/day group and 3 animals from the 300 mg/kg/day group were sacrificed after 6.5 months. Additionally, 3 animals from the 300 mg/kg/day group discontinued feeding at 6.5 months and recovered for 1.5 months, before being sacrificed.

[i] All animals were fed the substance for 6 months and then sacrificed, with the exception of 2 dogs from the high-dose group. These were fed the substance for four months, placed on control diets for 2 months, and then sacrificed with the other animals at 6 month.

[j] Pair-fed study.

examination showed atrophy of lymphoid tissue and liver degeneration and necrosis in all groups except the 900 mg/kg bw per day group (Inai et al., 1985).

4.3. Rats

4.3.1. Butyl-p-hydroxybenzoate (No. 5)

Butyl-*p*-hydroxybenzoate was dissolved in soybean oil at a ratio of 100 mg/0.5 ml and administered via oral incubation to rats for a period of 13–15 weeks at levels calculated to result in a daily intake of 0, 0.25, or 50 mg butyl-*p*-hydroxybenzoate/kg bw. The animals were divided into 2 groups: group 1 consisted of 41 animals and contained the control animals as well as those that received 0.25 mg/kg bw; group 2 consisted of 36 animals and contained those animals that received 50 mg/kg bw. Body weight was measured twice weekly, showing no significant difference between the experimental group and the control. Some animals were terminated on a predetermined schedule for histopathological evaluation, and all animals remaining alive at the end of the experiment were terminated. There were no sporadic mortalities and no significant histopathological differences between the treated groups and the control (Ikeda and Yokoi, 1950). Based on these data, the NOAEL is 50 mg/kg bw per day.

Wistar rats (12/sex/dose) were fed a powdered mixture of butyl-*p*-hydroxybenzoate and Purina™ Dog Chow at either 2000 or 8000 mg/kg bw per day for a period of 12 weeks. Control animals were fed powdered Dog Chow without the addition of the compound for a period of 12 weeks. Body weight and food intake were measured every 2 weeks and necropsy and histological examinations were performed at the end of the experiment. Animals found dead before the end of the study were necropsied and the appropriate tissues were fixed for histopathological evaluation. Although there were no effects in the low-dose group, all of the males and many of the females in the high-dose group died within several weeks of beginning the experiment. Additionally, body weight and motor activity decreased in these animals and they had a slower growth rate as compared to the control (Matthews et al., 1956).

4.3.2. p-Methoxybenzaldehyde (No. 13) and Piperonal (No. 45)

In 10 male and 10 female weanling rats, a mixture of eugenol (89.7 mg/kg bw), *p*-methoxybenzaldehyde (7.3 mg/kg bw), and piperonal (16.0 mg/kg bw) was incorporated into the diet at a level calculated to provide an average daily intake of 113 mg/kg bw for a period of 90 days. Food intake and growth were measured each week. After 12 weeks on the test material, the urine of 3 male and 3 female animals were tested for sugar, albumin, and hemoglobin levels. No sugar was present in any of the urine tested; however, the male rats tested positive for albumin. The authors did not consider the

increase in albumin to be pathologically significant. All surviving animals were terminated at 90 days, necropsies were performed, and organ weights were taken. Based on weekly measurement of body weight gain and food intake, hematological examinations, clinical chemistry determinations, and gross and histopathological examinations, there was no significant difference between test and control animals and no histopathology related to administration of the test mixture (Trubeck, 1958). Based on these data, the NOAEL was 7.3 mg/kg bw per day for *p*-methoxybenzaldehyde, and 16.0 mg/kg bw per day for piperonal.

Groups of 5 male and 5 female weanling Osborne-Mendel rats were given 50 mg/kg bw per day of *p*-methoxybenzaldehyde (No. 13) or piperonal (No. 45) in the diet for a period of 27–28 weeks or 500 mg/kg bw per day for a period of 15 weeks. Ten male and 10 female rats were fed the same diet without the test substance as a control. Body weight, food intake, and general condition were noted weekly. Hematological examinations were performed at the end of the study. No effects were seen in the animals at either dose level. Based on these results 500 mg/kg bw per day was determined to be the NOAEL for each substance, which is greater than 1000 times the estimated daily per capita intake ("eaters only")[5] of *p*-methoxybenzaldehyde or piperonal from use as a flavouring agent in the USA. (Hagan et al., 1967).

In a previous study, weanling Osborne-Mendel rats (number/group unspecified) were fed methyl salicylate in the diet at levels calculated to provide an average daily intake of 500 mg/kg bw and 50 mg/kg bw of methyl salicylate for 16 and 28 weeks, respectively. The authors observed no adverse effects in any of the rats at either dose level (Hagan et al., 1965).

4.3.3. Methyl-o-methoxybenzoate (No. 15)

Ten Fischer 344 rats (5 animals/sex/group; 28-days-old) were maintained on diets containing methyl *o*-methoxybenzoate (vehicle, corn oil) at a level calculated to provide an average daily intake of 94 mg/kg bw for a period of 14 days. The same number of control animals were fed only a corn oil-supplemented diet. Observations on mortality were made twice a day for the duration of the experiment. Weekly measurement of body weight and food consumption revealed no significant differences between test and control groups. At necropsy, liver and kidney weights were measured and histopathological examinations were performed. The only significant result was a decrease in relative kidney weight in the male animals that was not seen in the females. There was no difference, however, in the absolute liver and kidney weights of any of the animals and there was no evidence of kidney histopathology. The authors concluded that this change was not a treatment related response (Van Miller and Weaver, 1987). Based on these data, the authors reported a NOAEL of 94 mg/kg bw per day, which is greater than 100,000 times the estimated daily per capita intake ("eaters only")[5] of 0.1 μg methyl-*o*-methoxybenzoate/kg bw per day from use as a flavouring agent in the USA.

4.3.4. Vanillin (No. 22) and ethyl vanillin (No. 39)

The protocols used for the Hagan et al. *p*-methoxybenzaldehyde/piperonal studies cited above were instituted in studies using vanillin. In these studies, Osborne-Mendel rats were maintained on a diet containing vanillin at a level calculated to provide an average daily intake of 500 mg/kg bw for 16 weeks. In the Hagan study, an additional group of ten rats was fed 50 mg/kg bw per day of vanillin for 28 weeks. There were no effects seen at either of the dose levels in either study (Hagan et al., 1967).

Vanillin or ethyl vanillin was dissolved in corn oil and added to the diet of 5 male weanling Osborne-Mendel rats at levels calculated to provide an average daily intake of either 1000 or 2500 mg/kg bw for a period of 1 year. Ten (10) male and 10 female rats were fed a 3% corn oil diet as a control. Weekly measurement of body weight and food intake, and observations of general condition failed to show any differences between test and control groups. At necropsy, hematological examinations were performed. No effects were seen in any of the animals at any dietary levels (Hagan et al., 1967). The authors concluded that the NOAEL for vanillin and ethyl vanillin was 2500 mg/kg bw per day.

Four (4) groups of young albino rats (8 rats/group) were fed either vanillin or ethyl vanillin as a 4% solution in milk at an estimated daily intake level of either 20 mg/kg bw for 126 days or 64 mg/kg bw for 70 days. In the 70-day study, half the animals were terminated and the other half were put on a recovery diet for 8 more weeks. Additionally, 12 rats were given dose levels of 300 mg/kg bw of vanillin or ethyl vanillin as a 4% solution in olive oil orally by gavage 2 times per week for 14 weeks. Appearance, behavior, and body weight gain were noted for all animals and there were no significant effects observed with the exception of reduced growth rate and myocardial, renal, hepatic, lung, spleen, and stomach injuries (nature not specified) seen at the 64 mg/kg bw dose level (Deichmann and Kitzmiller, 1940).

Groups of CD Sprague-Dawley rats (20/sex/group) were fed ethyl vanillin at dose levels of 0, 500, 1000, or 2000 mg/kg bw per day for a period of 13 weeks (Hooks

[5] Intake (μg/d) calculated as follows: [[(annual volume, kg) × (1×10^9 mg/kg)]/[population × 0.6 × 365 days]], where population (10%, "eaters only") = 24×10^6 for the USA; 0.6 represents the assumption that only 60% of the flavor volume was reported in the survey (NAS, 1982, 1987) or as anticipated volume and 0.8 of the flavor volume was reported in Lucas et al. (1999). Intake (μg/kg bw/d) calculated as follows: [(μg/d)/body weight], where body weight = 60 kg. Slight variations may occur from rounding off.

et al., 1992). Food consumption and body weight were recorded weekly. Ophthalmoscopy was done before treatment and at the termination of the study. Detailed hematological and clinical chemical examinations were carried out at week 6 and 13. At termination, all animals were necropsied and organ weights recorded. A complete histological examination was performed on rats in the control and top dose groups. The examination was extended to the low and intermediate dosage groups where treatment-related effects were suspected.

There were some observed differences in food intake and body weight gain compared with controls, however the authors considered them unrelated to treatment because they were not dosage-related in magnitude and because of intragroup variability noted in the feeding patterns of all groups of male rats. There were no treatment-related differences from control in hematological parameters at week 6 or at termination (Hooks et al., 1992).

At autopsy, enlarge cervical lymph nodes were noted on males at the intermediate-dose group, and in both sexes at the highest dose group. In addition, there was a reduction in adipose tissue in rats of both sexes at the highest dose group. Absolute liver weights were similar to controls but relative liver weights were increased in the intermediate- and high-dose animals. An increased incidence of hepatic peribiliary inflammatory change in both males and females in the two highest dose groups and minor bile duct hyperplasia in a few of the intermediate and high-dose males. There were no changes observed in the liver parenchyma and degenerative or inflammatory changes of the bile duct epithelium. The authors concluded that no treatment-related changes were observed at 500 mg/kg bw per day (Hooks et al., 1992).

4.3.5. Methyl salicylate (No. 28)

A series of studies (Abbott and Harrisson, 1978) were performed to evaluate the intake of methyl salicylate on bone density. For a period of 11 weeks, 10 rats per group (equal numbers of males and females) were maintained on diets containing methyl salicylate at levels calculated to provide an average daily intake of 0 (control), 100, 180, 315, 565, or 1000 mg/kg bw. The test groups received the substance at 50% of the final dose level for the first two weeks, 75% for the third and fourth weeks, and 100% thereafter. Body weight and food consumption were measured weekly. The males at 315 mg/kg bw per day showed decreased weight gain when compared to the controls and the males and females at 565 and 1000 mg/kg bw per day showed both decreased weight gain and increased bone density at the metaphyses of the femur, humerus, tibia, and radius. No such bone changes were observed at lower dose levels.

In a 12-week study, two groups of 5 male rats were fed methyl salicylate in the diet at levels calculated to provide and average daily intake of 1000 or 300 mg/kg bw. No control animals were used. All of the animals in the high-dose group died during the first six weeks of the study and bone lesions were observed in full body X-rays of these animals. The animals in the low-dose group all survived to the end of the study and exhibited none of the bone lesions observed in the high-dose group (Abbott and Harrisson, 1978).

In a second 11-week study to re-evaluate a group of structurally related substances on bone density, methyl salicylate was administered in the diet at 1000 mg/kg bw per day to 15 rats. Twenty (20) rats served as negative controls. Whole body X-rays were taken periodically and body weights were checked. The experimental group showed 20% mortality, whereas the negative control animals all survived. Changes in bone density similar to those in the first study were reported for both methyl salicylate and acetylsalicylic acid, indicating that bone changes were associated with high-dose levels of ortho-hydroxybenzoic acid derivatives (salicylates) (Abbott and Harrisson, 1978).

In a six-week study, animals were fed ad libitum with a diet estimated to provide an average daily intake of 0, 300, or 1000 mg methyl salicylate/kg bw. In addition, paired feeding groups (10 groups) were administered diets providing the following intakes: 0, 300 mg methyl salicylate/kg bw per day, or 1000 mg methyl *p*-hydroxybenzoate/kg bw per day. All paired feeding animals received their daily rations equal to the mean daily amount of food consumed by the 1000 mg/kg bw per day ad libitum methyl salicylate group. The 300 mg/kg bw per day ad libitum group showed a slightly lower growth rate than the control, whereas the body weight changes for the paired feeding groups (negative control, 300 mg methyl salicylate/kg bw per day, and 1000 methyl *p*-hydroxybenzoate/kg bw per day) were similar to the body weight changes for the 1000 mg methyl salicylate/kg bw per day ad libitum group. Mortalities were seen in the high-dose methyl salicylate ad libitum group as well as all members (including the control group) of the pair fed study (Abbott and Harrisson, 1978).

Finally, a study was performed in which groups of 20 rats, divided evenly by sex, were fed either 300, 450, 600, or 1000 mg methyl salicylate/kg bw per day for a period of 11 weeks. This study was undertaken to evaluate the progression of bone change. A fifth group was used as a negative control and fed the basic diet without the addition of the test substance. Two (2) animals per group were X-rayed weekly and then terminated a week later until the end of the experiment. Histological examinations were done on some of the femurs of certain animals as well. The highest-dose group began showing bone changes at week 2, and the 600 mg/kg bw per day group began showing signs at week 5. The remaining groups gave negative X-rays throughout the 11-week

period. The histological analysis showed an increase in cancellous bone in the high-dose group at week 2 and in the second highest group by week 8 (Abbott and Harrisson, 1978).

In a 17-week study, 2 groups of 10 male and 10 female rats were maintained on a diet designed to result in an average daily intake of 50 or 500 mg/kg bw of methyl salicylate. Twenty (20) male and female rats were used as a negative control. Based on weekly measurement of body weight and organ weights at necropsy and the results of gross and microscopic examinations, no adverse effects were observed in either treatment group (Webb and Hansen, 1963).

Six (6) rats (equal numbers of male and female) were fed a diet of either 0 or 1000 mg methyl salicylate/kg bw per day. As each experimental animal died, one control animal was killed at the same time until all of the animals were dead. The first male died at 11 days, followed by the next 2 males at 19 days. The first female died at 31 days, then 40 days, and finally the last died at 71 days. The last animal to die was given X-ray and microscopic examinations. Aside from being lethal, the 1000 mg/kg bw per day dose cause increased bone density in the metaphysis of all bones, labored respiration, and gastric hemorrhages in the glandular stomach (Webb and Hansen, 1963).

4.3.6. Isoamyl salicylate (No. 32)

A study was conducted in which groups of Wistar rats (15 male and 15 female) were maintained on diets calculated to provide an average daily intake of 0, 2.5, 25, or 250 mg/kg bw of isoamyl salicylate for a period of 13 weeks. An additional group of 5 females and 5 males received only 0, 25, or 250 mg/kg bw per day for a period of 2 or 6 weeks. The animals were weighed at days 1, 2, 6, 9, and 13, and then weekly until day 91. Food and water consumption was measured for 24 h prior to weighing and urine during the last 2 days of treatment was collected and analyzed. At necropsy, organ weights were measured and hematology and histopathological examinations were performed. The highest dose groups for both sexes experienced increased relative kidney weight and males in the 25 mg/kg bw dose group experienced increased relative and absolute kidney weight, although without any associated histopathological changes. There was also a decrease in weight gain and food intake observed in the highest dose. In a separate paired feeding study in which two groups of 10 rats were fed either 0 or 250 mg methyl salicylate/kg bw per day for a period of 98 days, a decrease in body weight and food consumption was concluded by the authors to be due to the unpalatable nature of the diet. The NOAEL of 2.5 mg/kg bw per day is greater than 10,000 times the daily per capita intake ("eaters only")[5] of 0.1 μg isoamyl salicylate/kg bw per day for use as a flavoring ingredient in the USA (Drake et al., 1975). Table 3.

4.4. Rabbits

4.4.1. Vanillin (No. 22) and ethyl vanillin (No. 39)

Vanillin and ethyl vanillin were given as a solution in milk to 1 rabbit at 240 mg/kg bw per day for 56 days and 2 rabbits at the same dose level for 126 days. Both substances were also administered as a solution in 10% glycerol to rabbits. Vanillin was evaluated at 83 mg/kg bw per day for 14 days and 103 mg/kg bw per day for 61 days. Ethyl vanillin was evaluated at 15 mg/kg bw per day for 15 days, 15 mg/kg bw per day for 31 days, 32 mg/kg bw per day for 17 days, 41 mg/kg bw per day for 31 days, and 49 mg/kg bw per day for 49 days (Deichmann and Kitzmiller, 1940).

Appearance, behavior, and body weight gain were noted for all animals and there were no significant effects observed. There was no evidence of gross or histopathological alterations in test animals. In the glycerol solution group of rabbits receiving both substances, anemia, diarrhea and lack of weight gain were observed in the highest dose group and no toxic effects were seen at any of the lower dose groups. Glycerol poisoning, evidenced by restlessness, tremor, convulsion, and coma, was observed in rabbits given 83 mg vanillin/kg bw per day for 14 days and the rabbits given 15 mg ethyl vanillin/kg bw per day for 15 days. (Deichmann and Kitzmiller, 1940).

4.5. Dogs

4.5.1. Methyl salicylate (No. 28)

Four (4) groups of purebred beagles (3 males, 3 females) were fed methyl salicylate in capsule form at 150, 300, 500, or 800 mg/kg bw per day for a period of 7.5 months. The dogs were given one half of the dosage at the morning feeding and the other half in the afternoon for 6 days per week. A group of the same total number (2 male, 4 female) were fed the same diet without the addition of the substance and served as a negative control. After 6.5 months, 3 of the 300 mg/kg bw per day dogs, 2 of the 150 mg/kg bw per day, and all of the negative control animals were terminated and 3 of the 300 mg/kg bw per day animals were put on a recovery diet. After 7 months, all of the remaining animals were terminated, except the animals placed back on the normal diet, which were terminated after 8 months (Abbott and Harrisson, 1978).

Body weight was measured weekly and hematology examinations were performed at the fifth month on the two low-dose groups. After termination, the organ weights were taken, and gross and histological examinations were performed. All of the animals in the high-dose group were dead by the second week. Of the 500 mg/kg bw per day group, only 2 survived the duration of the experiment, while the rest died at weeks 2, 3, 5, and 8. For the two low-dose groups, body weight

Table 3
Mutagenicity/genotoxicity studies for hydroxy- and alkoxy-substituted benzyl derivatives used as flavoring ingredients in vitro

Substance name	Test system in vitro	Test object	Concentration/dose of substance[a]	Results	Reference
In vitro					
5. Butyl-*p*-hydroxybenzoate	Chromosome aberration test	Chinese hamster fibroblast cells	60 μg/ml	Negative[b]	Ishidate et al. (1984)
5. Butyl-*p*-hydroxybenzoate	Ames test[c]	*Salmonella typhimurium* TA92, TA1535, TA100, TA1537, TA94, TA98, TA2637	1000 μg/plate	Negative[d]	Ishidate et al. (1984)
5. Butyl-*p*-hydroxybenzoate	Ames test	*S. typhimurium* TA98, TA100	Up to 1000 μg/plate	Negative[d]	Haresaku et al. (1985)
6. Anisyl alcohol	Ames test[e]	*S. typhimurium* TA100	Up to 500 μg/plate	Negative[b]	Ball et al. (1984)
12. Veratraldehyde	Ames test	*S. typhimurium* TA1535, TA100, TA1537, TA15378, TA98	8000 μg/plate	Negative[d]	Nestmann et al. (1980)
12. Veratraldehyde	Ames test	*S. typhimurium* TA1535, TA100, TA1537, TA1538, TA98	8000 μg/plate	Negative[d]	Douglas et al. (1980)
12. Veratraldehyde	Mutation Assay	*Saccharomyces cerevisiae* D7, XV185-14C	Not reported	Negative[b]	Nestmann and Lee (1983)
12. Veratraldehyde	Ames test[c]	*S. typhimurium* TA1535, TA98, TA100, TA97, TA1537	Up to 6666 g/plate	Negative[d]	Mortelmans et al. (1986)
12. Veratraldehyde	Ames test	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100	1000 μg/plate[f]	Negative[d]	Heck et al. (1989)
12. Veratraldehyde	Mouse lymphoma forward mutation assay	Mouse lymphoma L5178Y cells	1400 μg/ml[f]	Positive[d]	Heck et al. (1989)
12. Veratraldehyde	Ames test[c]	*S. typhimurium* TA100, TA102, TA104, TA1538, TA982	Not reported	Negative[d]	Dillon et al. (1992)
12. Veratraldehyde	Ames test[c]	*S. typhimurium* TA100, TA102, TA104	33–3333 μg/plate	Negative[d]	Dillon et al. (1998)
12. Veratraldehyde	Unscheduled DNA synthesis assay	Rat hepatocyte cells	100 μg/ml[f]	Negative	Heck et al. (1989)
13. *p*-Methoxybenzaldehyde	Ames test[c]	*S. typhimurium* TA92, TA1535, TA100, TA1537, TA94, TA98, TA2637	5000 μg/plate	Negative[d]	Ishidate et al. (1984)
13. *p*-Methoxybenzaldehyde	Ames test	*S. typhimurium* TA98, TA100	Up to 500 μg/plate	Negative[d]	Kasamaki et al. (1982)
13. *p*-Methoxybenzaldehyde	Chromosome aberration test	Chinese hamster fibroblast cells	500 μg/ml	Negative[b]	Ishidate et al. (1984)
13. *p*-Methoxybenzaldehyde	Ames test[c]	*S. typhimurium* TA102, TA97	Up to 1000 μg/plate	Negative[d]	Fujita and Sasaki (1987)
13. *p*-Methoxybenzaldehyde	Rec assay	*Bacillus subtilis* H17, M45	22 μg/disk	Negative[b]	Oda et al. (1979)
13. *p*-Methoxybenzaldehyde	Ames test	*S. typhimurium* TA102	5000 μg/plate	Negative[d]	Müller et al. (1993)
13. *p*-Methoxybenzaldehyde	Ames test	*S. typhimurium* TA 100	Up to 1000 μg/plate	Negative	Rapson et al. (1980)
13. *p*-Methoxybenzaldehyde	Mouse lymphoma forward mutation assay	Mouse lymphomas L5178y	Up to 467 μg/ml	Negative	Wangenheim and Bolcsfoldi (1988)
			542–773 μg/ml	Positive[b]	Wangenheim and Bolcsfoldi (1988)
13. *p*-Methoxybenzaldehyde	Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537	408 μg/plate	Negative[d]	Florin et al. (1980)
13. *p*-Methoxybenzaldehyde	Chromosome aberration test	Chinese hamster cell line B241	0.0068 μg/ml	Positive[d]	Kasamaki et al. (1982)
13. *p*-Methoxybenzaldehyde	Mutation assay	Phage PM2	1362 μg/ml	Negative	Becker et al. (1996)
13. *p*-Methoxybenzaldehyde	SCE analysis	Human lymphocytes	Up to 272 μg/ml	Positive[b]	Jansson et al. (1988)
13. *p*-Methoxybenzaldehyde	DNA alkaline unwinding assay	Mouse lymphoma L5178Y/TK+/- cells	Up to 820 μg/ml	Negative[d]	Garberg et al. (1988)
			956–1093 μg/ml	Positive[d]	Garberg et al. (1988)
13. *p*-Methoxybenzaldehyde	SCE analysis	Chinese hamster ovary K-1 cells	Up to 14 μg/ml	Negative	Sasaki et al. (1987)
14. *p*-Ethoxybenzaldehyde	Ames test	*S. typhimurium* TA1535, TA100, TA1537, TA1538, TA98	3600 μg/plate	Negative[d]	Wild et al. (1983)
19. Methyl anisate	Paper disk mutation assay	*Escherichia coli* Sd-4-73	Not reported	Negative[b]	Szybalski (1958)
21. Vanillyl alcohol	SOS induction assay	*E. coli* PQ37	Not reported	Positive[b]	Ohshima et al. (1989)

22. Vanillin	Ames test	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100	10,000 μg/plate[f]	Negative[d]	Heck et al. (1989)
22. Vanillin	Rec assay	*B. subtilis* H17, M45	21 μg/disk	Negative[b]	Oda et al. (1979)
22. Vanillin	Chromosome aberration test	Chinese hamster fibroblast cells	1000 μg/ml	Negative[b]	Ishidate et al. (1984)
22. Vanillin	Ames test	*S. typhimurium* TA 1535, TA1537, TA1538, TA98, TA100	5000 μg/plate	Negative[d]	Pool and Lin (1982)
22. Vanillin	Ames test[c]	*S. typhimurium* TA1535, TA98, TA100, TA97, TA1537	Up to 10,000 μg/plate	Negative[d]	Mortelmans et al. (1986)
22. Vanillin	Paper disk mutation assay	*E. coli* Sd-4-73	Not reported	Negative[b]	Szybalski (1958)
22. Vanillin	Ames test	*S. typhimurium* TA98, TA100, TA1535, 1537, 1538	Not reported	Negative[d]	Nagabhushan and Bhide (1985)
22. Vanillin	Ames test	*S. typhimurium* TA92, TA1535, TA100, TA1537, TA94, TA98, TA2637	10,000 μg/plate	Negative[d]	Ishidate et al. (1984)
22. Vanillin	Ames test	*S. typhimurium* TA100	Up to 1000 μg/plate	Negative	Rapson et al. (1980)
22. Vanillin	Mouse lymphoma mutation assay	Mouse lymphoma L5178Y cells	Up to 1500 μg/ml	Negative[d]	Heck et al. (1989)
22. Vanillin	Mutation assay	*E. coli* CSH26/pYM3, CSH26/pSK 1002	Up to 15,215 μg/ml	Negative	Takahashi et al. (1990)
22. Vanillin	Ames test	*S. typhimurium* TA98, TA100	Up to 1000 μg/plate	Negative[d]	Kasamaki et al. (1982)
22. Vanillin	Chromosome aberration test	Chinese hamster B241 cells	Up to 0.006 μg/ml	Negative	Kasamaki and Urasawa (1985)
22. Vanillin	SCE assay	Human lymphocyte cells	0–152 μg/ml	Positive	Jansson et al. (1986)
22. Vanillin	Mitotic gene conversion assay	*S. cerevisiae*	10,000 μg/ml	Negative	Rosin (1984)
22. Vanillin	Chromosome aberration test	Chinese hamster V79 lung cells	15,215–152,150 μg	Negative[b]	Tamai et al. (1992)
			304,300 μg	Positive[b]	Tamai et al. (1992)
22. Vanillin	Chromosome aberration test	Human lymphocytes	Up to 608 μg/ml	Negative	Jansson and Zech (1987)
22. Vanillin	Chromosome aberration test	Chinese hamster cell line B241	0.003 μg/ml	Negative	Kasamaki et al. (1982)
22. Vanillin	SCE assay	Chinese hamster ovary K-1 cells	Up to 15 μg/ml	Negative	Sasaki et al. (1987)
22. Vanillin	SCE assay	Human lymphocytes	152–304 μg/ml	Positive	Jansson and Zech (1987)
22. Vanillin	UDS assay	Rat hepatocyte cells	500 g/ml	Negative	Heck et al. (1989)
22. Vanillin	SOS induction assay	*E. coli* PQ37	Not reported	Positive[b]	Ohshima et al. (1989)
22. Vanillin	Micronucleus assay	Human hepatoma (Hep-G2) cells	50 mg/ml	Negative	Sanyal et al. (1997)
			500 mg/ml	Positive	Sanyal et al. (1997)
22. Ethyl vanillin isobutyrate	Ames test	*S. typhimurium* TA1535, TA 1537, TA1538, TA98 and TA100	Up to 5000 μg/plate	Negative[d]	King and Harnasch (1997)
26. Salicylaldehyde	Mutation assay	*S. typhimurium* TA1535/pSK1002	111 μg/ml	Negative[d]	Nakamura et al. (1987)
26. Salicylaldehyde	Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537	366 μg/plate	Negative[d]	Florin et al. (1980)
26. Salicylaldhelyde	Ames test[c]	*S. typhimurium* TA98, TA100	Dose not reported	Negative[d]	Sasaki and Endo (1978)
26. Salicylaldehyde	SCE induction assay	Human lymphocyte cells	Up to 61 μg/ml	Negative[g]	Jansson et al. (1988)
28. Methyl salicylate	Chromosome aberration test	Hamster lung fibroblast cells	Not reported	Positive[b]	Kawachi et al. (1981a, 1981b)
28. Methyl salicylate	Rec assay	*B. subtilis* H17, M45	23 μg/disk	Negative[b]	Oda et al. (1979)
28. Methyl salicylate	Chromosome aberration test	Chinese hamster fibroblasts	250 μg/ml	Negative[b]	Ishidate et al. (1984)
28. Methyl salicylate	Ames test	*S. typhimurium* TA92, TA1535, TA100, TA1537, TA94, TA98, TA2637	10,000 μg/plate	Negative[d]	Ishidate et al. (1984)
28. Methyl salicylate	Ames test[c]	*S. typhimurium* TA1535, TA98, TA100, TA97, TA1537	Up to 333.3 μg/plate	Negative[d]	Mortelmans et al. (1986)
28. Methyl salicylate	Ames test	*S. typhimurium* TA100, TA98	Not reported	Negative[d]	Kawachi et al. (1981a, 1981b)
28. Methyl salicylate	Rec assay	*B. subtilis*	Not reported	Negative[d]	Kawachi et al. (1981a, 1981b)
28. Methyl salicylate	Chromosome aberration test	Human embryo fibroblast cells	Not reported	Negative[b]	Kawachi et al. (1981a, 1981b)

Table 3 (*continued*)

Substance name	Test system in vitro	Test object	Concentration/dose of substance[a]	Results	Reference
28. Methyl salicylate	SCE assay	Human embryo fibroblast cells	Not reported	Negative[b]	Kawachi et al. (1981a, 1981b)
28. Methyl salicylate	Mutation assay	Silkworm	Not reported	Negative[b]	Kawachi et al. (1981a, 1981b)
38. Vanillyl butyl ether	Ames test	*S. typhimurium* TA1535, TA100, TA1537, TA98	5000 μg/plate	Negative[d]	Watanabe and Morimota (1989)
38. Vanillyl butyl ether	Mutation assay	*E. coli* WP2 *uvr* A	5000 μg/plate	Negative[d]	Watanabe and Morimota (1989)
39. Ethyl vanillin	Ames test	*S. typhimurium* TA1535, TA100, TA1537, TA1538, TA98	Up to 3600 μg/plate	Negative[d]	Wild et al. (1983)
39. Ethyl vanillin	Rec Assay	*B. subtilis* H17, M45	21 μg/disk	Negative[b]	Oda et al. (1979)
39. Ethyl vanillin	Chromosomal aberration test	Chinese hamster fibroblast cells	250 μg/ml	Positive[b]	Ishidate et al. (1984)
39. Ethyl vanillin	Ames test[c]	*S. typhimurium* TA1535, TA98, TA100, TA97, TA1537	Up to 8000 g/plate	Negative[d]	Mortelmans et al. (1986)
39. Ethyl vanillin	Ames test	*S. typhimurium* TA92, TA1535, TA100, TA1537, TA94, TA98, TA2637	10,000 μg/plate	Negative[d]	Ishidate et al. (1984)
39. Ethyl vanillin	Mouse lymphoma forwards mutation assay	Mouse lymphoma L5178Y cells	Up to 1000 μg/ml	Negative[g]	Heck et al. (1989)
			800 μg/ml	Weak positive[b]	Heck et al. (1989)
39. Ethyl vanillin	Ames test[c]	*S. typhimurium* TA97, TA102	Up to 1000 μg/plate	Negative[d]	Fujita and Sasaki (1987)
39. Ethyl vanillin	Ames test	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100	10,000 μg/plate	Negative[d]	Heck et al. (1989)
39. Ethyl vanillin	UDS assay	Rat hepatocyte cells	199 μg/ml	Negative	Heck et al. (1989)
39. Ethyl vanillin	SCE assay	Human lymphocytes	Up to 332 μg/ml	Negative[b]	Jansson et al. (1988)
39. Ethyl vanillin	SCE assay	Chinese hamster ovary K-1 cells	Up to 17 μg/ml	Negative	Sasaki et al. (1987)
43. Piperonyl acetate	Ames test[c]	*S. typhimurium* TA1535, TA98, TA100, TA97, TA1537	Up to 3333 μg/plate	Negative[d]	Mortelmans et al. (1986)
43. Piperonyl acetate	Ames test	*S. typhimurium* TA1535, TA100, TA1537, TA1538, TA98	3600 μg/plate	Negative[d]	Wild et al. (1983)
45. Piperonal	Ames test[h]	*E. coli* WP2uvrAtrp$^-$	2400 μg	Negative[d]	Sekizawa and Shibamoto (1982)
45. Piperonal	Ames test	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100	10,000 μg/plate	Negative[d]	Heck et al. (1989)
45. Piperonal	Ames test	*S. typhimurium* TA98, TA100	0.05–5000 μg/plate	Negative[d]	Kasamaki et al. (1982)
45. Piperonal	Ames test	*S. typhimurium* TA1537, TA1538, TA98, TA100	Up to 5000 μg/plate	Negative[d]	White et al. (1977)
45. Piperonal	Rec assay	*B. subtilis* H17, M45	20 μg/disk	Negative[b]	Oda et al. (1979)
45. Piperonal	Ames test	*S. typhimurium* TA100, TA1535, TA98, TA1537, TA1538	2400 μg	Negative[d]	Sekizawa and Shibamoto (1982)
45. Piperonal	Ames test[c]	*S. typhimurium* TA1535, TA1537, TA98, TA100	Up to 10,000 μg/plate	Negative[d]	Haworth et al. (1983)
45. Piperonal	UDS assay	Rat hepatocyte cells	502 μg/ml	Positive	Heck et al. (1989)
45. Piperonal	Chromosome aberration test	Chinese hamster cell line B241	0.075 μg/ml	Positive	Kasamaki et al. (1982)
45. Piperonal	Chromosome aberration test	Chinese hamster cell line B241	Up to 0.15 μg/ml	Negative	Kasamaki and Urasawa (1985)
45. Piperonal	Rec assay	*B. subtilis* H17/M45	5000 μg/disk	Positive[b]	Sekizawa and Shibamoto (1982)
45. Piperonal	Mouse lymphoma forward mutation assay	Mouse lymphoma L5178Y cells	Up to 1000 μg/ml	Negative[d]	Heck et al. (1989)

Substance name	Test system in vivo	Test object	Concentration of substance	Results	Reference
In vivo					
14. *p*-Ethoxybenzaldehyde	Basc test	*Drosophila melanogaster*	751 μg/ml	Negative	Wild et al. (1983)
14. *p*-Ethoxybenzaldehyde	Micronuleus test	NMRI mice	Up to 1005 mg/kg bw	Negative	Wild et al. (1983)
22. Vanillin	Micronucleus test	Male BDF1 mice	500 mg/kg bw	Negative	Inouye et al. (1988)

39. Ethyl vanillin	Basc test	*D. melanogaster*	8309 μg/ml	Negative	Wild et al. (1983)
39. Ethyl vanillin	Micronucleus test	Male BDF1 mice	Dose not reported	Negative	Furukawa et al. (1989)
39. Ethyl vanillin	Micronucleus test	NMRI mice	1000 mg/kg bw	Negative	Wild et al. (1983)
43. Piperonyl acetate	Basc test	*D. melanogaster*	4855 μg/ml	Negative	Wild et al. (1983)
43. Piperonyl acetate	Micronucleus test	NMRI mice	Up to 970 mg/kg bw	Negative	Wild et al. (1983)
45. Piperonal	Dominant lethal assay	ICR/Ha Swiss mice	Up to 620 mg/kg bw[j]	Negative	Epstein et al. (1972)
45. Piperonal	Dominant lethal assay	ICR/Ha Swiss mice	1000 mg/kg bw[j]	Negative	Epstein et al. (1972)

[a] The dose listed is either the highest dose if the result was negative or the dose at which the maximum number of revertant colonies was observed for the positive.
[b] Without metabolic activation.
[c] Pre-incubation method.
[d] With and without metabolic activation.
[e] Plate incorporation method.
[f] Highest inactive dose or lowest active dose.
[g] With metabolic activation.
[h] With histidine substitution.
[i] Administered by intraperitoneal injection.
[j] Administered by oral gavage.

changes revealed no significant difference between test and control animals and hematological examinations gave normal values. Increased liver and kidney weights were seen at all doses. The increased organ size was not present in the animals of the recovery group (Abbott and Harrisson, 1978).

A further study was initiated using the same protocol. Dogs were fed capsules of methyl salicylate at dose levels of 50,100, or 167 mg/kg bw per day. The lower dose groups contained 8 dogs, divided evenly by sex, and the high-dose group contained 12 dogs, also of equal sex distribution. All animals were sacrificed at 6 months, except for 2 animals of each sex from the high-dose group, which along with 4 negative control animals, were placed on a recovery diet for 2 additional months. All measurements were the same as the preceding study, with the exception of organ weight, in which only liver and kidney weights were assessed. There were no adverse effects reported in any of the dose groups (Abbott and Harrisson, 1978). The NOAEL of 167 mg/kg bw per day is greater than 100 times the daily per capita intake ("eaters only")[5] of 740 μg/kg bw per day for use as a flavoring ingredient in the USA.

Six groups of 1 male and 1 female dog were each fed a capsule of methyl salicylate at 0 (control), 50, 100, 250, 500, 800, or 1200 mg/kg bw 6 days a week for a period of up to 59 days. At necropsy, gross and microscopic examination was performed on 1 of the 800 mg/kg bw dogs and both of the 250 mg/kg bw. The dogs in the high-dose group vomited 3–4 h after every dose throughout the entire study. The 500 mg/kg bw group dogs had diarrhea and weakness during the last 3–4 days of the study. Both of the highest dose animals and one of the 800 mg/kg bw showed marked fatty metamorphosis in the liver. The three highest-dose groups were all either killed in extremis or died within the first month of the experiment (Webb and Hansen, 1963).

4.6. Long-term studies of toxicity and carcinogenicity

4.6.1. Mice

4.6.1.1. Butyl-p-hydroxybenzoate (No. 5). Three (3) groups of 50 male and 50 female ICR/jcl mice (8-week-old) were fed pellets containing either 225, 450, or 900 mg butyl-*p*-hydroxybenzoate/kg bw per day for a period of 102 weeks. Fifty (50) male and 50 female mice were fed the basal diet and used as control. Daily intake was measured once a week for the first 30 weeks, once every other week for the following 20 weeks, and then once every 4 weeks until the end of the experiment. Body weight was measured once a week for the first 6 weeks, once every other week for the next 24 weeks, and then every 4 weeks thereafter for the duration of the experiment. Any animals that were found dead during the experimental period were necropsied. All animals that were still alive at the end of the experiment were

terminated and necropsied at week 106, and the tissues of all animals, regardless of time of death, were examined histologically and necropsied. Tumor incidence in any of the experimental groups was not significantly different than the control group (Inai et al., 1985). Based on these data, the NOAEL was determined to be 900 mg/kg bw per day, which is greater than 10,000 times the estimated daily per capita intake ("eaters only")[5] of 0.0004 μg butyl-*p*-hydroxybenzoate/kg bw per day from use as a flavoring ingredient in the USA.

4.6.2. Rats

4.6.2.1. Vanillin (No. 22) and Ethyl vanillin (No. 39). Vanillin or ethyl vanillin dissolved in propylene glycol was added to the diet of groups of rats (12 male/12 female) estimated to provide an average daily intake of 250, 500, or 1000 mg/kg bw for 2 years. Twenty (20) rats were fed 3% propylene glycol as a control. Weekly measurement of body weight and food intake, and observations of general condition failed to show any differences between test and control groups. At necropsy, hematological examinations were performed. No effects were seen in any of the animals at any dietary levels (Hagan et al., 1967). Based on these data, the NOAEL was determined to be 1000 mg/kg bw per day for both vanillin and ethyl vanillin, which is greater than 100 times the estimated daily per capita intake ("eaters only")[5] of 2505 μg vanillin/kg bw per day and greater than 1000 times the estimated daily per capita intake ("eaters only")[5] of 714 μg ethyl vanillin/kg bw per day from their use as flavoring ingredients in the USA.

4.6.2.2. Methyl salicylate (No. 28). Twenty-five (25) male and 25 female rats were fed a diet calculated to provide 0, 50, 250, 500, or 1000 (24 male, 26 female) mg/kg bw per day of methyl salicylate for a period of 2 years. The diet was prepared for the animals every 2 weeks, and an additional 10% of methyl salicylate was added to each mix in order to account for evaporation. The animals were weighed weekly. Hematological exams performed at 3, 11, 17, and 22 months on 10 rats from each group revealed normal values. Histopathology was assessed for the control and the two high-dose groups. Additionally, gross pathology, organ weights, lesions, bones, and muscles were assessed upon termination in each group. Fifty percent (50%) of the rats in the high-dose group died after week 8. Only 5 rats survived past week 20, and 1 rat survived past week 35, finally dying at week 49. In the 500 mg/kg bw per day group, there was an increase in absolute organ weights for the testes, heart, and kidney, as well as increased amount of cancellous bone in the metaphysis. The 250 mg/kg bw per day group showed gross pituitary lesions in 10 rats as well as malignant pituitary tumors in 1 male and 2 females. These were not observed at higher dose levels. The authors concluded that the NOAEL for the study was 50 mg/kg bw per day (Webb and Hansen, 1963).

Fifty (50) weanling albino rats (equally divided by sex) were maintained on a dry diet containing methyl salicylate at levels calculated to result in the average daily intake of 0, 35, or 105 mg/kg bw for a period of 2 years. Based on regular evaluation of growth, survival, food consumption, general physical condition, blood and urine analyses, and necropsy, no adverse effects could be associated with administration of the test substance (Packman et al., 1961). The NOAEL based on the results of this study was 105 mg/kg bw per day, which is greater than 100 times the daily per capita intake ("eaters only")[5] of 740 g methyl salicylate/kg bw per day for use as a flavoring ingredient in the USA.

4.7. Dogs

4.7.1. Methyl salicylate (No. 28)

A long-term study was conducted in which 2 male and 2 female Beagles per group were fed a capsule 6 days a week containing either 0, 50,150, or 350 mg/kg bw per day of methyl salicylate for a period of 2 years. The animals were weighed weekly and hematological exams were performed 3 times prior to the initiation of the experiment, and then at 2 weeks, 1, 3, and 6 months, 1 year and 2 years. Microscopic analysis was performed on the 3 surviving high-dose dogs. Organ weights were taken at necropsy and histology examinations were performed on select tissues. Excluding two of the animals that died of unrelated diseases, there were no mortalities in this study and all of the hematological values were normal. The two highest-dose groups both showed an enlarged liver and an increase in the size of the hepatic cells. The 150 mg/kg bw per day group showed growth retardation. The NOAEL based on the results of this experiment was 50 mg/kg bw per day (Webb and Hansen, 1963).

4.8. Genotoxicity studies

4.8.1. In vitro

The hydroxy- and alkoxy-substituted benzyl derivatives were non-mutagenic in all standard plate incorporation and/or pre-incubation Ames assays using *Salmonella typhimurium* strains TA92, TA94, TA97, TA98, TA100, TA102, TA104, TA1535, TA1537, TA1538, and TA2637, when tested at concentrations ranging up to the level of cytotoxicity or at ICH/OECD-recommended maximum test concentrations, both in the absence and presence of metabolic activation (S9 fraction) (White et al., 1977; Sasaki and Endo, 1978; Douglas et al., 1980; Florin et al., 1980; Kawachi et al., 1981a,b; Nestmann et al., 1980; Rapson et al., 1980; Kasamaki et al., 1982; Pool and Lin, 1982; Sekizawa

and Shibamoto, 1982; Haworth et al., 1983; Wild et al., 1983; Ball et al., 1984; Ishidate et al., 1984; Haresaku et al., 1985; Nagabhushan and Bhide, 1985; Mortelmans et al., 1986; Fujita and Sasaki, 1987; Heck et al., 1989; Watanabe and Morimota, 1989; Dillon et al., 1992, 1998; Müller et al., 1993). A mutation assay in *Salmonella typhimurium* strain TA1535/pSK1002, using *umu* gene expression as an endpoint, produced negative results with salicylaldehyde (No. 26) (Nakamura et al., 1987). Mutation or DNA repair assays in *Escherichia coli* strains WP2 *uvrA*, WP2s, CSH26/pYM3, CSH26/pSK1002, PQ37, or Sd-4-73 performed with methyl anisate (No. 19), vanillyl alcohol (No. 21), vanillin (No. 22), vanillyl butyl ether (No. 38), and piperonal (No. 45) (Szybalski, 1958; Sekizawa and Shibamoto, 1982; Watanabe and Morimota, 1989; Ohshima et al., 1989; Takahashi et al., 1990), and *Saccharomyces cerevisiae* strains D3, D4, D7, or XV185-14C performed with veratraldehyde (No. 12) (Nestmann and Lee, 1983) also produced negative results.

Mixed results were obtained with the hydroxy- and alkoxy-substituted benzyl derivatives in the Rec DNA repair assay using *Bacillus subtilis* strains H17 and M45, with both positive and negative results reported for piperonal (No. 45), while negative results were reported for *p*-methoxybenzaldehyde (No. 13), vanillin (No. 22), ethyl vanillin (No. 39), and methyl salicylate (No. 28) (Oda et al., 1979; Kawachi et al., 1981a,b; Sekizawa and Shibamoto, 1982). A number of the mixed results in the Rec assay were due to apparently laboratory-specific factors, and Oda et al. (1979) reported only negative results with some of the same compounds. This suggests that laboratory conditions played an important role in the outcome of these particular Rec assays. Unfortunately, the Oda et al. (1979) studies were reported in Japanese with English abstracts only and could not be fully evaluated for methodological or other differences. Alternatively, it was not clear, based on the concentrations used in each Rec test, if cytotoxicity played a factor in the results. Finally, in non-mammalian assays, no mutations were observed in the silkworm when tested with methylsalicylate (No. 28) (Kawachi et al., 1981a,b).

In vitro assays in isolated mammalian cells produced both negative and positive results for some of the hydroxy- and alkoxy-substituted benzyl derivatives. Mixed results were reported for *p*-methoxybenzaldehyde and vanillin in the sister chromatid exchange (SCE) assay in several Chinese hamster cell lines and in human lymphocytes (Jansson et al., 1986, 1988; Jansson and Zech, 1987; Sasaki et al., 1987). Negative results were obtained in this assay when ethyl vanillin, salicylaldehyde, and methyl salicylate were tested (Kawachi et al., 1981a,b; Sasaki et al., 1987; Jansson et al., 1988). In the chromosome aberration (ABS) assay, also performed in Chinese hamster and human cell lines, similarly mixed results were obtained with *p*-methoxybenzaldehyde, vanillin, ethyl vanillin, piperonal, and methyl salicylate (Kawachi et al., 1981a,b; Kasamaki et al., 1982; Ishidate et al., 1984; Kasamaki and Urasawa, 1985; Jansson and Zech, 1987; Tamai et al., 1992). The results in the SCE and ABS assays generally were obtained independently of the presence or absence of metabolic activation. Mixed, but mostly positive, results were obtained with veratraldehyde, *p*-methoxybenzaldehyde, and ethyl vanillin in the forward mutation assay in L5178Y mouse lymphoma cells, both with and without metabolic activation (Garberg et al., 1988; Wangenheim and Bolcsfoldi, 1988; Heck et al., 1989). Vanillin and piperonal were negative in the mouse lymphoma assay (Heck et al., 1989). Vanillin was weakly positive in the micronucleus test in human Hep-G2 cells, producing only a moderate response at the highest concentration tested (Sanyal et al., 1997). No unscheduled DNA synthesis (UDS) was observed in rat hepatocytes exposed to veratraldehyde, vanillin, or ethyl vanillin (Heck et al., 1989). Piperonal produced a positive UDS result in initial testing but the finding could not be repeated in subsequent tests (Heck et al., 1989). As a result, the activity of piperonal in this assay was questionable.

Numerous in vitro anti-mutagenicity assays were conducted with some of the hydroxy- and alkoxy-substituted benzyl derivatives and included evaluations in several sub-mammalian and mammalian cell lines. Positive anitmutagenic activity was reported for *p*-methoxybenzaldehyde, and ethyl vanillin (Ohta et al., 1986a,b; Imanishi et al., 1990; Ohta, 1995). Mixed results were reported for vanillin (Takahashi et al., 1990; Tamai et al., 1992; Sanyal et al., 1997). Analysis of the concentrations, test organisms, and study methods failed to reveal an explanation for the discrepant results from these studies. No anti-mutagenic effect was observed with piperonal, or methyl salicylate (Ohta et al., 1983, 1986a,b).

4.8.2. In vivo

The hydroxy- and alkoxy-substituted benzyl derivatives were inactive in all in vivo assays. In mammals, compounds were administered orally or by ip injection at doses that were significant fractions of the reported lethal dose levels. The micronucleus test was negative with *p*-ethoxybenzaldehyde (No. 14) at a dose of 1005 mg/kg bw, ethyl vanillin (No. 39) at a dose of 1000 mg/kg bw, vanillin (No. 22) at a dose of 500 mg/kg bw, and piperonyl acetate (No. 43) at a dose of 620 mg/kg bw (Wild et al., 1983; Hayashi et al., 1988; Furukawa et al., 1989). Piperonal (No. 45), administered by ip injection at 1000 mg/kg bw to test for mutagenicity using the modified dominant lethal assay, produced a very slight increase in early foetal deaths as compared to the incidence in control mice; however, the authors reported that the result was not statistically

significant and no similar finding was reported after administration by oral gavage (Epstein et al., 1972).

In the sex-linked recessive lethal mutation assay in fruit flies (*Drosophila melanogaster*), negative results were obtained for *p*-ethoxybenzaldehyde, ethyl vanillin, and piperonyl acetate, after feeding at concentrations of 751, 8309, and 4855 μg/ml, respectively (Wild et al., 1983). Vanillin (No. 22) produced an anti-mutagenic response in fruit flies, while both vanillin and *p*-methoxybenzaldehyde (No. 13) were anti-mutagenic in mice (Imanishi et al., 1990; Sasaki et al., 1990; de Andrade et al., 1992). The data on vanillin, including the in vitro results, suggest some anti-mutagenic activity with this compound, although the relevance of this finding is questionable and impossible to extrapolate to the low exposures that are likely through flavor use in food.

4.8.3. Conclusion

The hydroxy- and alkoxy-substituted benzyl derivatives did not produce mutagenic activity in bacterial or other submammalian cellular systems. Mixed results were obtained in the bacterial Rec assay and in clastogenicity assays in isolated mammalian cells. These findings likely reflect to some degree the activity of alcohols or aldehydes in biological systems since they were obtained both with and without metabolic activation, and cytotoxicity often was a limitation at high test concentrations. More importantly, the positive in vitro activity was not translated into mutagenic, clastogenic, or genotoxic activity in the in vivo test systems. These latter negative findings were obtained in several species and indirectly encompass, based on metabolic considerations, additional members of the family of benzyl derivatives. As a result, it is concluded that the hydroxy- and alkoxy-substituted benzyl derivatives do not have in vivo genotoxic potential.

4.9. Other relevant studies

4.9.1. Reproduction

4.9.1.1. Estrogenic activity

4.9.1.1.1. Butyl 4-hydroxybenzoate (No. 5). In an in vitro study designed to examine the estrogen receptor binding affinities of a structurally diverse group of chemicals, 10^{-5}–10^{-3} M butyl 4-hydroxybenzoate (butyl paraben) was incubated with estrogen receptors (ER) isolated from uterine homogenates of mature female Sprague-Dawley outbred rats in a competitive binding assay with [^{3}H]-estradiol (Blair et al., 2000). As for other alkyl esters of 4-hydroxybenzoic acid, butyl 4-hydroxybenzoate was defined as a weak estrogen receptor binder. Compared to the potent ER-binding properties of diethylstilbestrol {mean IC_{50} (50% inhibition of [^{3}H]-estradiol binding) value of $2.25 \times 10^{-10} \pm 0.05 \pm 10^{-10}$ M and relative binding affinity (RBA) of 399.5%, logRBA = +2.60}, butyl 4-hydroxybenzoate showed a mean IC_{50} of $1.05 \times 10^{-4} \pm 0.35 \pm 10^{-4}$ M and a relative binding affinity (RBA) of 0.0009% (logRBA = −3.07). The authors describe logRBA values of less than −2 as weak binders (Blair et al., 2000).

Additional in vitro evidence for the ER binding properties of butyl paraben was assessed using estrogen sensitive MCF7 human breast cancer cells. In a single point competitive binding assay, ERs from MCF7 human breast cancer cells were incubated with [^{3}H]-estradiol. Although [^{3}H]-Estradiol binding was inhibited 86 and 49%, by 1,000,000 and 100,000 fold molar excess of butylparaben, respectively, only a 10-fold molar excess of 17-β-estradiol resulted in a 100% inhibition. Since ERs function as ligand-activated transcription factors, an additional study was conducted to determine if ER binding of parabens could alter regulation of estrogen sensitive gene expression. A clonal line of MCF7 cells was transfected with the ERE-CAT estrogen regulated reporter gene. In the presence of 10^{-5} M butylparaben, expression of the reporter gene was increased 1.2-fold over 24 h and 1.1-fold over 7 days. In order to show similar receptor gene expression, the concentrations of butylparaben (10^{-5} M) were three to five orders of magnitude greater than that of 17-β-estradiol (10^{-10}–10^{-7} M range). The authors noted that butylparaben induced maximal proliferation at 10^{-5} but was highly cytotoxic at $(1–2) \times 10^{-4}$ (Byford et al., 2002).

Butyl 4-hydroxybenzoate incubated with MCF-7 breast cancer cells induced maximal cell proliferation at a concentration of 2×10^{-5} M (Okubo et al., 2001). Gene expression of ERα and progesterone receptor (PR) following incubation of butylparaben with MCF-7 cells was monitored by tracking RNA expression. The authors observed a slight decrease in the RNA encoding ERα at 48 h and a gradual 5-fold increase of PR expression at 24 h compared to control levels. In an in vitro competitive binding assay, butylparaben showed weak non-specific binding to both ERα and ERβ (Okubo et al., 2001).

In a competitive binding assay with [^{3}H]-estradiol, butylparaben showed weak binding affinity to ER in cytosolic extract of immature rat uterine cells (i.e., 5 orders of magnitude less than diethylstilbestrol) (Routledge et al., 1998). In an in vivo uterotrophic assay immature female rats were administered butyl paraben orally (4, 40, 400, 800 and 1200 mg/kg bw per day) and by subcutaneous injection (40, 80, 400, 600, 800, 1000, and 1200 mg/kg bw per day). The rats receiving butyl paraben orally showed a slight but not significant increase in wet and dry uterine weight at the 800 and 1200 mg/kg bw per day doses. The rats administered butylparaben sc showed a significant ($P < 0.05$, $P < 0.01$) increase in wet and dry uterine weights at doses greater than 400 mg/kg bw per day with the dose of 1200 mg/kg bw per day showing a 170% increase in uterine weights (Routledge et al., 1998). The significant

difference in activity based on route of administration may be due, in part, to the intestinal hydrolysis of butylparaben given by the oral route.

In an in vivo study to assess the effects of butylparaben on the reproductive systems of male mice, ICR mice were maintained on diets containing 0, 0.01, 0.10 or 1.00% butylparaben daily for 10 weeks (Oishi, 2002). These dietary levels correspond to average daily food intake calculated from food consumption of 14.4 ± 3.60, 146 ± 3.60 and 1504 ± 357 mg/kg bw per day, respectively. There were no treatement related effects from butylparaben in the diet to the liver, ventral prostates, seminal vesicles, or preputial glands. The absolute and relative weights of the epididymides were significantly ($P < 0.05$) greater in the highest dose group (1%) when compared to controls. A dose-dependent decrease of elongated spermatid counts was observed in stages VII–VIII seminiferous tubules. These stages have maximal requirement for testosterone. A significant decrease ($P < 0.05$) in round spermatids at the 1% level was not evident at lower dietary levels. In addition, spermatic counts were lower in all of the treated groups. There was a significant decrease in the serum testosterone levels but only at the 1% dietary level. (Oishi, 2002).

In a study to assess estrogenic effects of butyl paraben on immature male Wistar rats, the animals were maintained on diets designed to provide 0, 0.01, 0.10, and 1.00% butylparaben in the diet for 8 weeks (Oishi, 2001). These dietary levels correspond to average daily food intake calculated from food consumption of 0, 10.4 ± 3.07, 103 ± 31.2, and 1026 ± 310 mg/kg bw, respectively. Body weights and food consumption were comparable among test and control groups. Absolute weights of the epididymides and the seminal vesicles with coagulation glands were significantly lower for the 1% group. There was a dietary level-dependent decrease in the relative epididymides weights at 0.1% and higher. The 1% group showed a decreased sperm count which was 58.2% of controls. An intake level-dependent deterioration of testicular sperm indices was observed. Daily sperm production was reduced in all test groups receiving butyl paraben in the diet. The serum testosterone levels was also decreased in a dietary concentration dependent fashion and reached significance at 0.1% ($P < 0.05$) (Oishi, 2001).

Several in vitro and in vivo studies described above have demonstrated estrogenic activity of high-dose levels of alkyl esters of *p*-hydroxybenzoic acid (parabens), including butyl *p*-hydroxybenzoate (No. 5). These studies have reported that butyl *p*-hydroxybenzoate can bind with rodent uterine estrogen receptors (Routledge et al., 1998; Blair et al., 2000) and MCF7 human breast cancer cells (Byford et al., 2002); regulate gene expression in endogenous estrogen regulated genes pS2 and progesterone receptors in breast cancer cells (Routledge et al., 1998) and in transfected estrogen-regulated reporter genes in yeast and human breast cancer cells (Routledge et al., 1998; Byford et al., 2002) at concentration levels approaching cytotoxicity (10^{-4}). At dietary levels providing intakes in excess of 1000 mg/kg bw, butylparaben administration results in decreased testosterone levels, related decreases in spermatid counts in spermatogenic stages VII–VIII, and epidydimal weights in male mice and rats. However, these effects must be considered in the context of the extremely low level of use of butyl *p*-hydroxybenzoate used as a flavoring ingredient and the metabolic fate in humans following oral ingestion. In light of the weak estrogenic activity of high-dose levels of butyl paraben in rodents and the low intake of this substance as a flavoring substance, it is highly unlikely that humans will experience any reproductive effects from use of butyl paraben as a flavoring substance.

4.9.1.1.2. Veratraldehyde (No. 12), vanillin (No. 22), ethyl vanillin (No. 39), and piperonal (No. 45). Four (4) groups of 10 virgin Crl CD rats were administered each of the four substances by gavage once daily, for 7 days prior to cohabitation, through cohabitation (maximum of 7 days), gestation, delivery, and a 4-day postpaturition 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, number of offspring per litter). Offspring indices included daily observation, clinical signs, examination for gross external malformations, and measurement of body weight. The only consistent 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 effects observed in the offspring at any dose level.

Specifically, dams given 80, 400, or 800 mg/kg bw per day of verataldehyde showed decreased body weights and body weight gain at the low- and mid-dose. At the high dose, increased mortality and gross lesions were reported. Animals given 125, 250, or 500 mg/kg bw per day of vanillin exhibited a slight non-significant decrease in food consumption and body weight at the mid-dose and a statistically significant ($P < 0.05$) decrease in body weight and increase in clinical signs at the high dose. Animals given 200, 1000, or 2000 mg/kg bw per day of ethyl vanillin exhibited a slight non-significant increase in body weight and a statistically significant ($P < 0.05$) decrease in food consumption at the low dose. The mid- and high-dose group exhibited mortality, gross lesions, clinical signs and depressed body weight gain and food consumption. Animals given 250, 500, or 1000 mg/kg bw per day of piperonal exhibited a slight non-significant decrease in body weight at the mid-dose and statistically significant ($P < 0.05$) decrease in body weight, a decrease in food consumption, increased mortality and decreased fertility index at the high dose. The only effect

to offspring was decreased viability and body weight gain at the high-dose level of piperonal. Based on the lack of adverse effects to offspring at all dose levels and to dams at the low-dose level for each substance, the authors concluded that there were no reproductive or developmental effects (Vollomuth et al., 1990).

4.9.1.1.3. Methyl salicylate. Mice. Methyl salicylate (methyl 2-hydroxybenzoate) was tested by the NTP using a Fertility Assessment by Continuous Breeding (FACB) in CD-1 mice (NTP, 1984a,b). In the initial study groups of male and female mice (F_0 generation) were administered 0, 25, 50, or 100 mg/kg bw per day of methyl 2-hydroxybenzoate via corn oil gavage during a 7-day pre-mating period, throughout a 98-day cohabitation period and a 21-day segregation period. Methylsalicylate administration had no effect on the number of pairs able to produce at least one litter, the number of letters per pair, the number of live pups per pair or the proportion or sex of pups born alive. As a continuation of the study 1 or 2 female and male pups (F_1 generation) weaned from control and high-dose were randomly selected and when they were approximately 90-days-old, they were mated to produce the F_2 generation. There were no significant effects on mating behavior, fertility rate, or reproductive performance. It was concluded that up to 100 mg/kg bw per day methyl salicylate was not a reproductive toxicant (NTP, 1984a).

Four (4) groups of 11-week-old CD-1 mice (40 males and 40 females in control group; 20 males and 20 females in all treatment groups) were given 0 (vehicle control), 100, 250, or 500 mg methyl salicylate/kg bw per day via corn oil gavage. All mice were treated for 7-days prior to, 100 days during, and 21 days following cohabitation, which provided a total treatment period of 18 weeks. Reproductive performance was assessed by observing the number of litters per breeding pair, live pups per litter, proportion of pups born alive, sex of pups born alive, and live pup weight. Measurements of body weight were also taken at weeks 1, 2, 3, 6, 10, 14, and 18. The only significant result was a slight decrease in the mean number of litters in the highest-dose group ($P < 0.05$). In a crossover mating trial, high-dose males were mated with control females and high-dose females were mated with control males. Two attempts to determine the affected sex were unsuccessful due to poor fertility of the control groups in follow-up experiments. Eleven (11) animals died during the 18 weeks of this study, with deaths distributed relatively evenly among the 4 treatment groups. Gavage trauma was the most common cause of death and none of the deaths were considered chemical or dose related (NTP, 1984b). The NTP concluded that methyl salicylate "*does interfere with reproduction in CD-1 mice*" without further elaboration.

Rats. Methyl salicylate was tested in three-generation studies. In one three-generation study, male and female Osborne-Mendell rats were fed 0, 500, 1500, 3000 or 5000 ppm methyl salicylate in the diet 100 days prior to mating (Collins et al., 1971). These dietary concentrations were calculated to provide estimated daily intakes of 0, 25, 75, 150 or 250 mg/kg bw (FDA, 1993), respectively. The parental rats were mated twice to produce two litters (F_{1a} and F_{1b} generations). From these F_2 and F_3 generations were produced. There was no effect on fertility index at any dose. In the F_1 generation at the two highest dietary concentrations, there was a decrease in average litter size, survival, and average number of live-born per female. External examination revealed no gross abnormalities. The significant changes reported for the F_1 generations were not seen in the F_2 generation although there was a decreasing trend observed. There were no visible abnormalities and necropsy of the F_2 generation showed no effects. A supplemental study using F_{2b} rats was conducted to test the efficacy of calcium on any adverse effects produced by methyl salicylate. Rats from each dose level were co-administered 1500 ppm calcium carbonate (approximately 600 ppm available as calcium) and were mated. The first and second litters were observed as described above. Supplementation of calcium did not appear to alleviate or enhance reported effects.

In another three-generation study, groups of male and female Wistar rats were fed diets containing up to 0.5% methyl salicylate for 60 days after which the rats were mated to produce F_1 generation (Abbott and Harrisson, 1978). At 4 months of age, male and female F_1 rats were randomly selected to produce the F_2 generation. No gross abnormalities were reported in any of the litters. No significant differences among treated and control groups were reported for any reproductive parameters including mating performance, number of stillborn, viability, mean litter size, number born, number live-born and number alive at 5 days.

4.9.1.1.4. 2,4-Dihydroxybenzoic acid (No. 36). Following s.c. injection of 380 mg/kg 2,4-dihydroxybenzoic acid at day 9 of pregnancy to adult female Sprague-Dawley rats (10/group), there were no differences from the control group in total number of implants, mean fetus weight, number of live fetuses, resorptions or malformed fetuses. (Koshakji and Schulert, 1973). Male and female Sprague-Dawley rats were given a subcutaneous injection of 430 mg/kg bw of 2,4-dihydroxybenzoic acid and plasma and serum calcium levels were measure 3 h after treatment. No change in plasma or serum calcium levels was observed. Pregnant female rats were given a 428 mg/kg bw dose of 2,4-dihydroxybenzoic acid subcutaneously accompanied by a second 214 mg/kg bw dose 2 h later. No change in serum calcium was observed after the initial dose, but after the second dose decreased calcium levels and high serum 2,4-dihydroxybenzoic acid levels were recorded. Females sacrificed on day 21 of gestation showed no significant change in number of litters, implantations, resorptions, or dead fetuses. Increased

malformations (kinky tail) and fetotoxcity (33%) was reported (Saito et al., 1982). No statistical analysis was presented to support these claims. The authors concluded that the toxicities induced by dihydroxybenzoic acid derivatives were distinctly different from the salicylic acid-induced abnormality.

4.10. Human

4.10.1. Methyl salicylate

Six clinicians were requested to conduct a retrospective study of 155 cases of juvenile arthritis in which children were treated with salicylates and periodic X-rays were available to determine whether any bone density changes had occurred during treatment. Patients were given a wide variety of salycilates and, depending on the age and weight of the patient, doses were in the range of 100–3240 mg per day. The duration of treatment was anywhere from several months to intermittent dosage for a period of 14 years. Upon review, it was the unanimous conclusion of the reporting clinicians that no accumulation of cancellous bone was seen as the result of salycilate therapy in children (Abbott and Harrisson, 1978).

5. Recognition of GRASr status

The group of hydroxy and alkoxy-substituted benzyl 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 1978, the Panel evaluated the available data and affirmed the GRAS status of these flavor ingredients (GRASa). In 1993, the Panel initiated a comprehensive program to re-evaluate the status of all FEMA GRAS flavor ingredients concurrent with a systematic revision of the FEMA Scientific Literature Reviews (SLRs). The group of benzyl derivatives was reaffirmed as GRAS (GRASr) based, in part, on their self-limiting properties as flavoring substances in food; their rapid absorption, metabolic detoxication, and excretion in humans and other animals; their low level of flavor use; the wide margins of safety between the conservative estimates of intake and the no-observable-adverse effect levels determined from subchronic and chronic studies and the lack of significant genotoxic and mutagenic potential. This evidence of safety is supported by the fact that the intake of hydroxy- and alkoxy-substituted benzyl derivatives as natural components of traditional foods is greater than their intake as intentionally added flavoring substances.

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