EUGENOL AND RELATED HYDROXYALLYLBENZENE DERIVATIVES

First draft prepared by

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of seven hydroxyallylbenzene flavouring agents (Table 1), including eugenol (No. 1529). The evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, p. 170). The Committee has evaluated one member of the group previously: eugenol (No. 1529) was evaluated at the twenty-sixth meeting (Annex 1, reference 59), when an ADI of 0–2.5 mg/kg bw was assigned.

Three of the seven flavouring agents (Nos 1527, 1529 and 1531) have been reported to occur naturally in various foods. They have been detected in wheaten bread, clove buds, leaves and stems, oregano, tarragon, dill, basil, rosemary, pimento leaf and berry, cinnamon bark and leaf, laurel, apples, cherries, whisky and red and white wine (Nijssen et al., 2004).

Table 1. Summary of results of safety evaluations of eugenol and related hydroxyallylbenzene derivatives

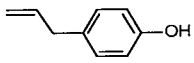
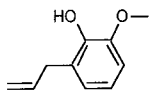
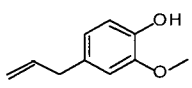
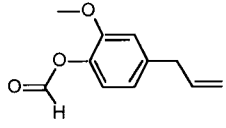
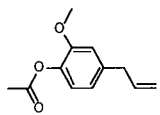
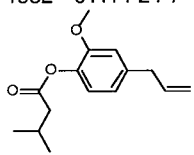
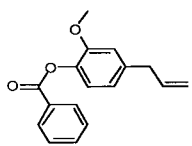
Flavouring agent	No.	CAS no. and structure	Step A3[a] Does intake exceed the threshold for human intake?	Step A4 Is the substance or its metabolites endogenous?	Step A5 Adequate NOEL for substance or related substance?	Comments	Conclusion based on current intake
Structural class I							
4-Allylphenol	1527	501-92-8 	No Europe: 0.09[a] USA: 0.2[a]	NR	NR	See notes 1, 2 and 3	No safety concern (conditional)
2-Methoxy-6-(2-propenyl)phenol	1528	579-60-2 	No Europe: 0.1[a] USA: 0.2[a]	NR	NR	See notes 1, 2 and 3	No safety concern (conditional)
Eugenol	1529	97-53-0 	Yes Europe: 1107 USA: 3364	No	Yes. The NOEL of 300 mg/kg bw per day (National Toxicology Program, 1983) is > 16 000 and 5000 times the estimated daily intakes of 18 µg/kg bw in Europe and 56 µg/kg bw in the USA, respectively, when eugenol is used as a flavouring agent.	See notes 1, 2 and 3	An ADI of 2.5 mg/kg bw was established for eugenol (Annex 1, reference 59), which was maintained at the present meeting.
Eugenyl formate	1530	10031-96-6 	No Europe: 0.01 USA: 0.05	NR	NR	See notes 1, 2, 3 and 4	No safety concern

Table 1 (contd)

Flavouring agent	No.	CAS no. and structure	Step A3[a] Does intake exceed the threshold for human intake?	Step A4 Is the substance or its metabolites endogenous?	Step A5 Adequate NOEL for substance or related substance?	Comments	Conclusion based on current intake
Eugenyl acetate	1531	93-28-7 	No Europe: 23 USA: 90	N/R	N/R	See notes 1, 2, 3 and 4	No safety concern
Eugenyl isovalerate	1532	61114-24-7 	No Europe: 0.4[a] USA: 0.5[a]	N/R	N/R	See notes 1, 2, 3 and 4	No safety concern (conditional)
Eugenyl benzoate	1533	531-26-0 	No Europe: 0.003 USA: 0.9	N/R	N/R	See notes 1, 2, 3 and 4	No safety concern

CAS, Chemical Abstracts Service; N/R, not required for evaluation because consumption of the substance was determined to be of no safety concern at Step A3 of the Procedure.

Step 1: All the agents in this group are in structural class I (Cramer et al., 1978).

Step 2: All the agents in this group can be predicted to be metabolized to innocuous products.

[a] The threshold for human intake for structural class I is 1800 μg/day. All intake values are expressed in μg/day. The combined per capita intake of the flavouring agents in structural class I is 1130 μg per day in Europe and 3456 μg per day in the USA.

[b] Intake estimate based on anticipated annual volume of production

Table 1 (contd)

Notes:
1. The phenolic hydroxyl group forms a conjugate with glucuronic acid and is readily excreted in the urine.
2. Minor amounts of epoxide are formed on the allyl moiety, which undergoes hydrolysis, followed by conjugation and subsequent excretion.
3. Formation of quinone methide may occur, followed by conjugation with glutathione.
4. The ester group is hydrolysed by carboxyl esterases.

1.2 Estimated daily per capita exposure

Annual volumes of production have been reported for four of the seven flavouring agents in this group (Nos 1529–1531 and 1533). For the remaining three substances (Nos 1527, 1528 and 1532), anticipated annual volumes of production have been given for their proposed use as flavouring agents. The total reported and anticipated annual volumes of production of eugenol and the six related hydroxyallylbenzenes is about 7925 kg in Europe (International Organization of the Flavor Industry, 1995) and 26 227 kg in the USA (National Academy of Sciences, 1982, 1987; Lucas et al., 1999). Approximately 98% of the total reported and anticipated annual volume of production in Europe and approximately 97% of that in the USA is accounted for by eugenol (No. 1529). The estimated per capita exposure to eugenol is 1107 μg/day in Europe and 3364 μg/day in the USA. The estimated per capita exposure to all the other flavouring agents in the group (Nos 1527, 1528, 1530–1533), on the basis of reported or anticipated annual volumes of production, is 0.003–23 μg/day in Europe and 0.05–90 μg/day in the USA (National Academy of Sciences, 1982, 1987; International Organization of the Flavor Industry, 1995; Lucas et al., 1999), most of the values being at the lower end of the ranges. The estimated per capita exposure to each flavouring agent is reported in Table 2.

1.3 Absorption, distribution, metabolism and elimination

In humans and rodents, orally administered eugenol and related allylhydroxyphenol derivatives are rapidly absorbed from the gastrointestinal tract and efficiently extracted by the liver, where they mainly undergo phase II conjugation. The resulting glucuronide and sulfate conjugates are subsequently excreted in the urine. To a lesser extent, eugenol is metabolized to polar products, some of which are more reactive than the parent molecule. These products are also conjugated and eliminated, primarily in the urine. Minute amounts (< 1%) of eugenol are excreted unchanged. The primary urinary metabolites of the other agents containing an unsubstituted phenolic group also form glucuronic acid and sulfate conjugates. Eugenyl esters are hydrolysed to eugenol and the corresponding carboxylic acid. These metabolites are readily excreted, primarily in the urine.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

In applying the Procedure to flavouring agents for which both a reported and an anticipated volume of production were given, the Committee based its evaluation on the reported volume of production if the intake estimated from it exceeded the intake estimated from the anticipated volume of production, and applied no conditions

Table 2. Annual volumes of production of eugenol and related hydroxyallylbenzene derivatives used or proposed for use as flavouring agents in Europe and the USA

Agent (No.)	Reported[a] / anticipated annual volume (kg)	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
4-Allylphenol (No. 1527)					
Europe[e]	0.6	000	0.001		
USA[e]	0.6	0.1	0.002	+	NA
2-Methoxy-6-(2-propenyl)phenol (1528)					
Europe[e]	1	0.1	0.002		
USA[e]	1	0.2	0.003	–	NA
Eugenol (No. 1529)					
Europe	7 761	1107	18		
USA	25 537	3364	56	139 422	5
Eugenyl formate (No. 1530)					
Europe	0.1	000	0.0002		
USA[f]	0.3	000	0.001	–	NA
Eugenyl acetate (No. 1531)					
Europe	159	23	0.4		
USA	680	90	1	19 228	28
Eugenyl isovalerate (No. 1532)					
Europe[e]	3	0.4	0.007		
USA[e]	3	0.5	0.009	–	NA
Eugenyl benzoate (No. 1533)					
Europe	0.02	0.003	0.00005		
USA[f]	5	0.9	000	–	NA
Total					
Europe	7 925				
USA	26 227				

NA, not available; ND, no intake data reported; + reported to occur naturally in foods (Njissen et al., 2004), but no quantitative data; –. not reported to occur naturally in foods

[a] From International Organization of the Flavor Industry (1995) and Lucas et al. (1999) or National Academy of Sciences (1970, 1982, 1987)

[b] Intake (µg/person per day) calculated as follows: [(annual volume, kg) x (1 x 10^9 µg/kg)/(population x survey correction factor x 365 days)], where population (10%, 'eaters only') = 32 x 10^6 for Europe and 26 x 10^6 for the USA; where survey correction factor = 0.6 for Europe and 0.8 for the USA, representing the assumption that only 60% and 80% of the annual flavour volume, respectively, was reported in poundage surveys (International Organization of the Flavor Industry, 1995; Lucas et al., 1999; National Academy of Sciences, 1982) or in the anticipated annual volume.
Intake (µg/kg bw per day) calculated as follows: [(µg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[c] Quantitative data for the USA reported by Stofberg and Grundschober (1987)

[d] The consumption ratio is calculated as follows: (annual consumption from food, kg)/(most recent reported volume as a flavouring substance, kg)

Table 2 (contd)

[e] The volume cited is the anticipated annual volume, which was the maximum amount of flavour estimated to be used annually by the manufacturer at the time the material was proposed for flavour use. National surveys (National Academy of Sciences, 1970, 1982, 1987; Lucas et al., 1999), if applicable, revealed no reported use as a flavour agent.

[f] Annual volume reported in previous surveys in the USA (National Academy of Sciences, 1970, 1982)

to its decision on safety. If the intake estimated from the anticipated volume of production exceeded the intake estimated from the reported volume of production, the Committee based its evaluation on the anticipated volume of production but considered its decision on safety to be 'conditional', pending receipt of information on use levels or poundage data by December 2007. In applying the Procedure to flavouring agents for which only anticipated volumes of production were given, the decision was likewise made conditional.

Step 1. In applying the Procedure, the Committee assigned all seven agents (Nos 1527–1533) to structural class I (Cramer et al., 1978).

Step 2. All the flavouring agents in this group are predicted to be metabolized to innocuous products. The evaluation of all the agents in this group therefore proceeded via the A-side of the procedure.

Step A3. The estimated daily per capita exposure to six of the seven flavouring agents in structural class I is below the threshold of concern (i.e. 1800 μg/day). Three of these six substances (Nos 1530, 1531 and 1533) are reported to be used as flavouring agents; according to the Procedure, use of these three agents raises no safety concern at the estimated daily exposure. The other three substances (Nos 1527, 1528 and 1532) are proposed for use as flavouring agents. Although, according to the Procedure, use of these three agents raises no safety concern at the exposure estimated on the basis of the anticipated annual volumes of production, less uncertain exposure estimates are needed. The estimated daily exposure to the remaining agent in this group, eugenol (No. 1529), which is 1107 μg per person per day in Europe and 3364 μg per person per day in the USA, exceeds the threshold of concern for class I. Accordingly, the evaluation of eugenol proceeded to step A4.

Step A4. Eugenol and its metabolites are not endogenous. Accordingly, the evaluation of this agent proceeded to step A5.

Step A5. At its twenty-sixth meeting, the Committee established an ADI of 0–2.5 mg/kg bw per day for eugenol on the basis of the results of a 19-week study in rats (Annex 1, reference 59). At its current meeting, the Committee considered the results of a bioassay in rodents (National Toxicology Program 1983), in which the NOEL was 300 mg/kg bw per day. This NOEL for eugenol, which is consistent with the previous evaluation leading to the ADI, is more than 16 000 and 5000 times the estimated daily exposure to eugenol from its use as a flavouring agent in Europe (18 μg/kg bw) and the USA (56 μg/kg bw), respectively. The Committee therefore concluded that eugenol would not present a safety concern at the estimated daily exposure.

Considerations on exposure and other information used to evaluate eugenol and the six related hydroxyallylbenzene derivatives according to the Procedure are summarized in Table 1.

1.5 Consideration of secondary components

One member of this group of flavouring agents, eugenyl formate (No. 1530), has an assay value of < 95%. The secondary component in No. 1530, eugenyl formate, is eugenol (No. 1529), which was evaluated at the present meeting and considered not to present a safety concern at estimated current levels of exposure.

1.6 Consideration of combined exposure from use as flavouring agents

In the unlikely event that all seven agents in structural class I were to be consumed concurrently on a daily basis, the estimated combined exposure would exceed the human exposure threshold for class I (i.e. 1800 μg per person per day). All seven agents in this group are, however, expected to be efficiently metabolized and would not saturate metabolic pathways. Moreover, combined exposure to all seven agents would be well below the ADI of 0–2.5 mg/kg bw for eugenol. Overall evaluation of the data indicates that combined exposure would not raise concerns about safety.

1.7 Conclusions

The Committee maintained the previously established ADI of 0–2.5 mg/kg bw for eugenol. It concluded that use of the flavouring agents in the group of eugenol and related hydroxyallylbenzene derivatives would not present a safety concern at the estimated exposure. For three flavouring agents (Nos 1527, 1528 and 1532), the evaluation was conditional because the estimated exposure was based on anticipated annual volumes of production. The conclusion of the safety evaluation of these three agents will be revoked if use levels or poundage data are not provided by December 2007. The Committee noted that the available data on the toxicity and metabolism of the hydroxyallylbenzenes are consistent with the results of the safety evaluation with the Procedure.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

The relevant background information summarizes the key scientific data applicable to the safety evaluation of eugenol and six related hydroxyallylbenzene derivatives used as flavouring agents (see Table 1).

2.2 Additional considerations on exposure

Quantitative data on natural occurrence and consumption ratios have been reported for two agents in this group, eugenol (No. 1529) and eugenyl acetate (No. 1532), which indicate that exposure occurs predominantly from traditional foods (i.e. consumption ratio > 1) (Stofberg & Kirschman, 1985; Stofberg & Grunschober, 1987) (Table 2). The exposure to eugenol and eugenyl acetate from consumption of

traditional foods exceeds their intakes as added flavouring substances by a factor of at least 10 000 (Stofberg & Kirschman, 1985; Stofberg & Grunschober, 1987).

2.3 Biological data

2.3.1 Biochemical data

(a) Hydrolysis

This group includes four eugenyl esters that are anticipated to undergo ester hydrolysis in gastric juice and intestinal fluid. In general, aromatic esters are hydrolysed in vivo through the catalytic activity of carboxylesterases, the most important of which are the A-esterases (Heymann, 1980). Carboxylesterases are found in the endoplasmic reticulum of most mammalian tissues (Hosokawa et al., 2001); however, they occur predominantly in hepatocytes (Heymann, 1980; Graffner-Nordberg et al., 1998; Hosokawa et al., 2001).

Eugenyl acetate undergoes rapid hydrolysis to eugenol and acetic acid within 20 min of incubation with rat hepatocytes (100%), hepatic microsomes (100%), blood (87%) or plasma (72%) and with human hepatic microsomes (79–91%), blood (100%) and plasma (86%) (Castro et al., 2004). The maximum velocities and binding constants listed in Table 3 indicate that eugenyl acetate has similar rates of hydrolysis in rodents and humans (see Figure 1).

In a study of the hydrolysis of the structurally related ester, phenyl acetate[1], with pig liver carboxylesterase, the K_m and V_{max} values for phenyl acetate were reported to be 0.43 mmol/l and 438 μmol/min per mg protein, respectively, at a substrate concentration of 0.2–3 mmol/l phenyl acetate (Junge & Heymann, 1979). A second phenolic ester, *ortho*-tolyl acetate (*ortho*-methylphenyl acetate) was 60% hydrolysed in vitro after incubation with pancreatin for 2 h at 37 °C (Grundschober, 1977). Phenyl 2-hydroxybenzoate (phenyl salicylate) was hydrolysed to phenol and 2-hydroxybenzoic acid in humans (Fishbeck et al., 1975). A male volunteer was given one 90-mg capsule of phenyl salicylate per hour for 8 h, and urine was collected every 8 h for 72 h after the first dose. Analysis of total urinary phenol (phenol and its conjugates) showed a peak of 472 ppm (5 mmol/l phenol) during the second collection period; the level of free urinary phenol peaked at 25 ppm (0.3 mmol/l phenol) during the same period. By 60 h after the first dose, both total and free urinary phenol levels had returned to baseline levels (8 and 1 ppm, respectively).

Table 3. Kinetics of the hydrolysis of eugenyl acetate

Subcellular fraction	V_{max} (nmol/min per mg protein)	K_m (μmol/l)	V_{max}/K_m (ml/min)
Rat hepatic microsomes	3829	96.6	39.6
Human male hepatic microsomes	3656	88.6	41.3
Human female hepatic microsomes	2748	51.9	52.9

From Castro et al. (2004)

1

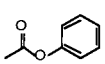
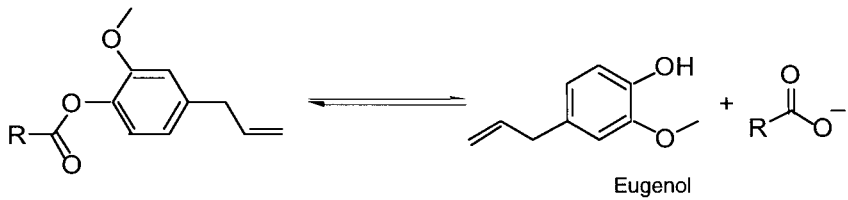
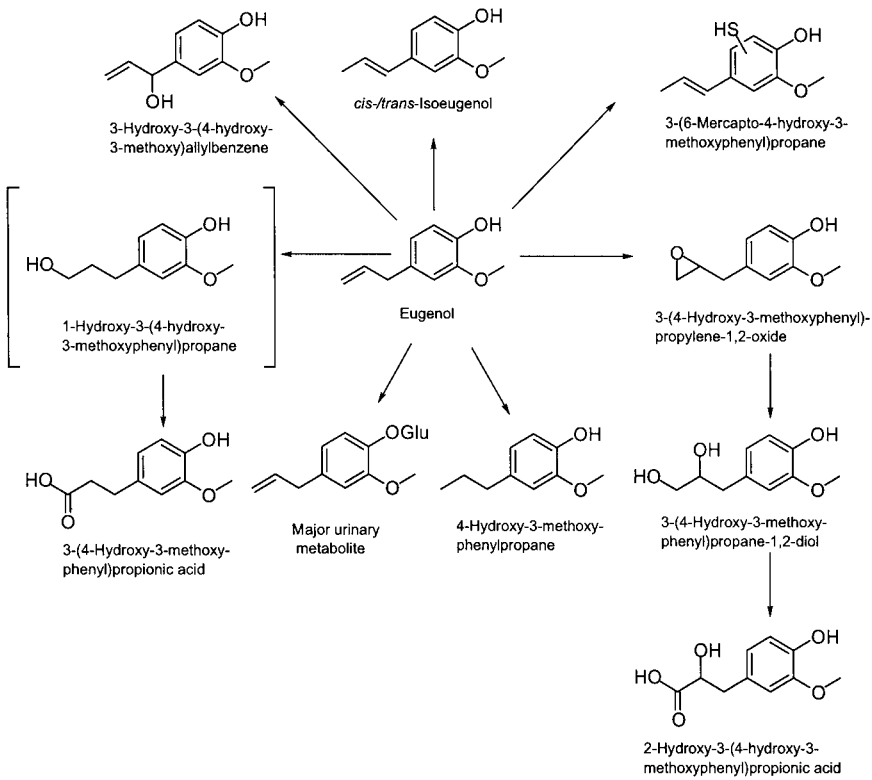


Figure 1. Hydrolysis of eugenyl esters



These findings for eugenyl acetate itself and structurally related acetate esters of phenol derivatives indicate that eugenyl esters (eugenyl formate, eugenyl acetate, eugenyl isovalerate and eugenyl benzoate) are rapidly hydrolysed in simulated intestinal fluid, hepatocytes and blood to form eugenol and the corresponding carboxylic acid (see Figure 2). Once hydrolysed, the resulting aromatic phenols and carboxylic acids are readily absorbed and metabolized by well-recognized biochemical pathways.

Figure 2. Human metabolism of eugenol



From Fischer et al. (1990)

(b) Absorption, distribution and elimination

In humans and rodents, orally administered eugenol and related allylhydroxyphenol derivatives are rapidly absorbed from the gastrointestinal tract and undergo mainly phase-II conjugation and subsequent excretion in the urine. To a lesser extent, eugenol is metabolized to polar products, which are also conjugated and eliminated primarily in the urine. Minute amounts (< 1%) of eugenol are excreted unchanged. The main urinary metabolites of eugenol are the glucuronic acid and sulfate conjugates of the phenolic hydroxyl group.

Four healthy male and four female volunteers (weighing 52–86 kg) were given three gelatine capsules, each containing 50 mg of eugenol (total dose, 150 mg; 1.7–2.9 mg/kg bw) with a normal breakfast (tea and two biscuits). Urine was collected 3, 6, 12 and 24 h after administration, and venous blood was sampled at 0, 15, 20, 25, 30, 40, 50, 60, 80, 100 and 120 min. In all body fluids analysed, eugenol was found predominantly in the conjugated form. Within 3 h, 71.3% (mean value for three volunteers) of the 150-mg dose was accounted for in the urine as conjugated eugenol or conjugated metabolites of eugenol. After 6 and 24 h, > 87% and 94%, respectively, of the dose had been excreted in the urine (Fischer et al., 1990).

Like humans, rodents also rapidly absorbed, metabolized and excreted eugenol given orally or by intraperitoneal injection. An unspecified number of female Wistar rats were given 0.5, 5, 50 or 1000 mg/kg bw of ^{14}C-ring-labelled eugenol in trioctanoin by stomach tube. More than 75% of the administered radiolabel was present in pooled 72-h urine, while 10% was found in pooled faeces. The 24-h urine contained mainly glucuronic acid and sulfate conjugates, the sulfate conjugates predominating at low doses and the glucuronic acid conjugates at 1000 mg/kg bw (Sutton et al., 1985).

Excretion of 50 mg/kg bw of ^{14}C-eugenol was essentially complete within 24 h in four female Wistar rats treated by intraperitoneal administration and in eight female Fischer 344 rats given the compound in trioctanoin by gavage. Excretion in the urine (91.2 ± 4.3% and 75.1 ± 9.4% for the intraperitoneal and oral routes, respectively) far exceeded that in the faeces (3.9 ± 1.6% and 7.4 ± 5.0%, respectively). The pattern of absorption and excretion observed was similar to that in mice. Eight CD-1 mice given 50 mg/kg bw of eugenol by intraperitoneal injection excreted 76.3 ± 4.1% and 4.9 ± 2.7% in 24-h urine and faeces, respectively (Sutton, 1986).

Rapid distribution to all organs, with tissue concentrations reaching 10–20 ng/mg of tissue, was observed in male Wistar rats given a single dose of 450 mg/kg bw ^{14}C-eugenol by intraperitoneal injection. Higher levels of radioactivity were reached in circulating erythrocytes than in sera, which showed a significant reduction in radioactivity 4 h after dosing. Less than 1% of the total radioactivity administered was eliminated as exhaled $^{14}CO_2$ (Weinberg et al., 1972).

When 500 mg of eugenol in sesame oil were administered to rats by gavage (about 1250 mg/kg bw), the compound was detected in the stomach, intestines and faeces, with lesser amounts in the liver and kidneys. Similar results were obtained after 1500, 2500 or 5000 mg of eugenol in sesame oil (about 750, 1250 and 2500 mg/kg bw, respectively) were administered to rabbits by gavage. Eugenol was detected mainly in the stomach, intestines and urine of rabbits at all dose, and in the lungs, liver, kidneys, muscle and blood of animals at 2500 mg/kg bw (Schröder & Vollmer, 1932).

Groups of eight male Donyu rats were given a single oral dose of 0 or 200 mg eugenol (about 500 mg/kg bw) in olive oil, and urine was collected at 12-h intervals. The 0–12-h and 12–24-h urine samples contained more glucuronides (45.4 ± 13.2 and 42.9 ± 9.1 mg of total glucuronic acid/12 h per rat) than the 0–12-h urine sample from control rats (10.9 ± 4.9 mg/12 h per rat). The excess amount of glucuronides was considered to be due to excretion of eugenol glucuronides. Therefore, orally administered eugenol undergoes rapid glucuronic acid conjugation and excretion in rats (Yuasa, 1974).

In conclusion, eugenyl esters are hydrolysed to eugenol and the corresponding carboxylic acid. Eugenol is then absorbed and undergoes rapid first-pass phase-I and -II metabolism in the liver. These metabolites are readily excreted, mainly in the urine and, to a lesser extent, in faeces.

(c) Metabolism

Eugenol and other hydroxyallylbenzene derivatives have several metabolic options for detoxication. The results of studies in humans indicate that most eugenol is rapidly conjugated with glucuronic acid or sulfate (Sutton, 1986; Fischer et al., 1990). To a much lesser extent, eugenol undergoes (1) isomerization to yield isoeugenol, which can then undergo allylic oxidation and reduction of the double bond; (2) epoxidation of the allyl double bond to yield an epoxide, which is hydrolysed to the corresponding diol and, subsequently, can be oxidized to the corresponding lactic acid derivative; (3) conjugation of glutathione (GSH) with a quinone-methide-type intermediate and (4) hydroxylation at the allyl position to yield 1′-hydroxyeugenol. As all these metabolites have a free phenolic OH group or other polar oxygenated functional groups, they readily conjugate with glucuronic acid or sulfate and are excreted in urine. In humans, 95% of ingested eugenol is excreted in conjugated form in the urine within 24 h (Fischer et al., 1990).

The metabolism of eugenol was investigated in four healthy male and four female volunteers, who received a 150-mg oral dose of eugenol in three gelatin capsules, each containing 50 mg of eugenol, after a normal breakfast. Within 24 h, > 55% of the administered dose had been excreted in the urine as the glucuronic acid or sulfate conjugate of eugenol. Other conjugated metabolites identified in the urine included *cis*- and *trans*-isoeugenol (7%), formed by isomerization of the double bond; 3-(4-hydroxy-3-methoxyphenyl)propane, formed by reduction of either eugenol or isoeugenol; and 3-(4-hydroxy-3-methoxyphenyl)propionic acid (4.6%), presumably formed by allylic hydroxylation of isoeugenol, followed by NADH-dependent enzymatic reduction of the double bond. Conjugated metabolites formed from epoxidation of eugenol included eugenol epoxide (1.6%), the corresponding diol (3%) and the 2-hydroxypropionic acid derivative, 2-hydroxy-3-(4-hydroxy-3-methoxyphenyl)propionic acid (3.3%), formed by oxidation of the diol primary alcohol. Tentatively identified metabolites included a thiophenol (11%) metabolite, presumably formed by GSH conjugation at an aromatic ring position, and a trace (< 1%) of 1′-hydroxyeugenol, formed by allylic hydroxylation at the benzylic position. Conjugated urinary metabolites accounted for 95% of the administered dose, while unconjugated eugenol accounted for > 0.1% (see Figure 2). The authors concluded that eugenol undergoes rapid first-pass conjugation and rapid elimination, only a small fraction participating in isomerization, epoxide-diol, GSH conjugation or benzylic hydroxylation pathways. They indicated that the short residence time of eugenol in the body might

explain the absence of toxic effects in numerous studies on eugenol (Fischer et al., 1990).

Two male volunteers (weighing 93 and 95 kg) received 0.6 mg of ^{14}C-eugenol (about 6.4 μg/kg bw) in the form of a gelatin capsule, which was taken orally with water. Within 24 h, 94–103% of the radioactivity was accounted for in the urine; none was found in faeces. Over 85% of the radioactivity in 24-h urine was accounted for by glucuronic acid and sulfate conjugates of eugenol, the glucuronic acid conjugates predominating. Minor amounts (2% each) of the corresponding diol 3-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol and alcohol 3-(4-hydroxy-3-methoxyphenyl)propane-2-ol were also detected. Unlike other hydroxyallylbenzene derivatives, which generally undergo oxidative metabolism at the allyl moiety, eugenol undergoes reductive metabolism and its conjugates are rapidly eliminated, which could explain its lack of toxicity (Sutton, 1986).

In rodents, the metabolic fate of eugenol appears to be similar to that in humans. The 24-h urine of eight female Wistar rats given 0.5, 5, 50 or 1000 mg/kg bw of ^{14}C-ring-labelled eugenol in trioctanoin by stomach tube contained glucuronic acid and sulfate conjugates of eugenol, the *O*-demethylation metabolite, 3,4-dihydroxypropylbenzene and the reduced metabolite, 3-methoxy-4-hydroxypropylbenzene. At the three lower doses, sulfate conjugates were the main metabolites, while at the highest dose glucuronic acid conjugates predominated. No reduction or demethylation metabolites (i.e. 3,4-dihydroxypropylbenzene and 3-methoxy-4-hydroxypropylbenzene) were detected at the highest dose (Sutton et al., 1985; Sutton, 1986).

To investigate the origin of the reduction and *O*-demethylation metabolites, 10 mg of ^{14}C-eugenol were incubated with rat caecal contents under anaerobic conditions. Formation of both reduction and *O*-demethylation metabolites suggested that the gut microflora are involved. Furthermore, the fact that no *O*-demethylation metabolites were found when eugenol was administered to germ-free Fischer 344 or Wistar rats pre-treated with antibiotics supports the conclusion that reduction and *O*-demethylation are mediated by gut microflora in rats (Sutton, 1986).

In a study to determine species-specific metabolism, 50 mg/kg bw of ^{14}C-eugenol were administered by gavage to female Wistar rats or injected intraperitoneally to CD-1 mice. Analysis of the urine showed that mice and rats excreted > 80% as glucuronic acid and sulfate conjugates. Mice excreted 27 ± 2.7% as sulfate conjugates and 53 ± 3.5% as glucuronic acid conjugates, while rats excreted 55 ± 3.3% as sulfate conjugates and 25 ± 3.8% as glucuronic acid conjugates (Sutton, 1986).

In a study of time-dependent changes in hepatic UDP-glucuronosyltransferase (UDPGT) activity, groups of eight male Donyu rats were given a single dose of 0 or 200 mg of eugenol (about 500 mg/kg bw) orally. Rats were killed at 12-h intervals up to 72 h, with collection of 12-h urine from two rats per group just before sacrifice. Excretion of *O*-glucuronic acid conjugates (ether glucuronides) peaked at 24 h, and excretion of total glucuronic acid conjugates peaked during the first 24 h after administration. Body weights, liver weights and hepatic UDPGT activity were measured. UDPGT activity increased between 12 and 48 h, maximal activity being observed at 48 h (0.277 ± 0.030 μmol/min per mg protein when compared with controls (0.169 ± 0.035 μmol/min per mg protein). The increase in UDPGT activity was accompanied by an increase in the relative weight of the liver, which is indicative

of a slow inductive adaptation of UDPGT to the large bolus dose of eugenol. Most of the UDPGT activity in the liver was localized in the microsomal fraction (Yuasa, 1974).

The studies in humans indicate that the majority of an oral dose of eugenol is conjugated with glucuronic acid or sulfate and excreted in the urine. Minor metabolic pathways include oxidation to the corresponding epoxide, followed by hydrolysis to the diol and then further oxidation; isomerization, followed by allylic oxidation and then reduction; and reduction of the alkene or 1′-hydroxylation (trace). All these metabolites have a free phenolic OH and are readily conjugated and excreted in the urine. Essentially all ingested eugenol (> 95%) is excreted as glucuronic acid or sulfate conjugates in the urine. The metabolism in rodents (mainly rats) is qualitatively and quantitatively similar to that in humans.

Conjugation of eugenol

Various studies have been undertaken to characterize more completely each of the metabolic pathways used by eugenol. Given the importance of phase-II glucuronic acid conjugation in the overall metabolism of eugenol, studies have been performed in vitro and in vivo–in vitro on UDPGT activity. In a study involving inhalation of eugenol, olfactory mucosa, olfactory bulb homogenate and brain tissue homogenate from male Wistar rats were incubated with 1 mmol/l of eugenol. The olfactory mucosa showed more glucuronic acid conjugation activity than olfactory bulb homogenate and brain tissue homogenate (Leclerc et al., 2002).

In general, phenolic hydroxyl moieties are poor substrates for UDPGT 1.4 protein but are excellent substrates for UDPGT 1.6 or 1.7 proteins (Wooster et al., 1993; Senafi et al., 1994; Green & Tephly, 1996). Cloned human bilirubin UDPGT 1.4 protein had low catalytic activity towards eugenol (9 pmol/min per mg protein), with a high K_m (1570 μmol/l) and a low V_{max} (4.8 pmol/min per mg protein) (Green & Tephly, 1996).

In a study of the heterogeneity of hepatic microsomal UDPGT activity, strains of rats with active (Wistar) and reduced (Gunn) UDPGT activity, as well as guinea-pigs, were studied. UDPGT activity was induced by either phenobarbital or 3-methylcholanthrene. Groups of four Wistar or Gunn rats were given either 80 mg/kg bw of phenobarbital or vehicle (saline solution) by intraperitoneal injection daily for 4 days. Groups of Wistar rats also received a single dose of 80 mg/kg bw of 3-methylcholanthrene or vehicle (corn oil) on day 1 only. All rats were killed on day 5. Groups of four male guinea-pigs received 20–40 mg/kg bw of phenobarbital daily for 9 days and were then killed. Liver microsomes isolated from all three groups of animals were incubated with 0.175–0.25 mmol/l eugenol, and UDPGT activity was measured. In Wistar rats, UDPGT activity increased approximately twofold with phenobarbital treatment and threefold with 3-methylcholanthrene treatment as compared with the activities of the respective control groups. The ratio of UDPGT activity in Gunn to Wistar rats was 0.6 for the control group and 1.2 for the phenobarbital-treated group, demonstrating the enhancement of UDPGT activity with phenobarbital pre-treatment. UDPGT activity increased approximately twofold in phenobarbital-treated guinea-pig livers incubated with 0.175 mmol/l eugenol. In a previous study, when eugenol (0–0.5 mmol/l) was incubated with male Wistar rat liver microsomes to assay UDPGT activity, there was a 1-min latency, which disappeared after activation with Triton X-100 (Boutin et al., 1983).

Oxidation and glutathione conjugation

Studies have been undertaken to evaluate the involvement of cytochrome P450 (CYP450) oxidation and subsequent GSH conjugation in the metabolism of eugenol. Most of these experiments were intended to evaluate the mechanism of action and potential toxicity of eugenol.

When male ddY mice were treated with 400 or 600 mg/kg bw of eugenol in olive oil by gavage, there was no evidence of hepatotoxicity, as indicated by the absence of changes in relative liver weight, liver blood volume and serum alanine aminotransferase (ALT) activity. Mice receiving 4 mmol/kg bw of the GSH inhibitor buthionine sulfoximine (BSO) by intraperitoneal injection 1 h before administration of 400 or 600 mg/kg bw of eugenol, however, showed significant increases in relative liver weights, serum ALT activity and volume of blood in the liver (indicative of hepatic congestion) 3 h after administration of 600 mg/kg bw in comparison with a control group receiving saline or olive oil. Additionally, the mortality of rats treated with BSO and 600 mg/kg bw eugenol was increased, although not statistically significantly. Gross examination showed marked enlargement and uniform or spotted dark-reddish colouration of the livers of mice receiving BSO and 600 mg/kg bw eugenol. Histologically, the centrilobular sinusoidal spaces were congested and vacuolation was observed near Glisson capsule. The livers of mice that survived 24 h after treatment showed marked necrosis in the centrilobular region. The livers of mice receiving eugenol at 600 mg/kg bw alone or BSO alone showed no liver pathological changes (Mizutani et al., 1991).

The effect of microsomal P450-dependent monooxygenase inhibitors on the hepatotoxicity of a high dose of eugenol (600 mg/kg bw) was evaluated in mice treated with the CYP450 inhibitors carbon disulfide methoxsalen or piperonyl butoxide together after administration of BSO. Treatment with carbon disulfide (50 mg/kg bw) resulted in complete protection against the hepatotoxicity seen in mice pre-treated with BSO and then given eugenol. Methoxsalen (50 mg/kg bw) partially prevented the increase in serum ALT activity reported after combined BSO and eugenol treatment, and it completely protected against increases in relative liver weight and hepatic congestion. Piperonyl butoxide (400 mg/kg bw) also suppressed BSO–eugenol-induced hepatotoxicity, although not as effectively as the other two inhibitors (Mizutani et al., 1991).

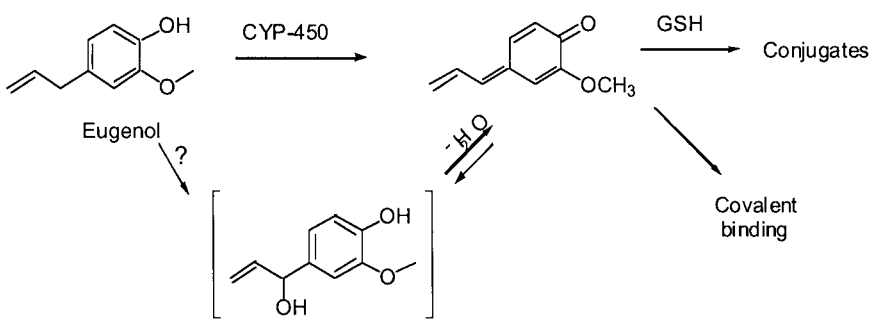
Pre-treatment of mice with phenobarbital, an inducer of CYP450 enzymes, enhanced the toxicity of eugenol administered in combination with BSO, increasing the relative liver weights and causing hepatic congestion and increased serum ALT activity. In the absence of BSO, phenobarbital-pre-treated mice showed no significant increases in any of the indicators of hepatotoxicity after dosing with 400 mg/kg bw of eugenol. Pre-treatment of mice with the CYP450 inhibitor b-naphthoflavone prevented an increase in serum ALT activity in mice treated with BSO and subsequently with 400 mg/kg bw eugenol; however, none of the indicators of hepatotoxicity was suppressed at 600 mg/kg bw of eugenol. The authors suggested that b-naphthoflavone acts by stimulating detoxicating pathways of eugenol metabolism at lower doses of this compound. These results suggest that a metabolite of eugenol formed by a CYP450-mediated reaction conjugates with GSH (Mizutani et al., 1991).

In a study of metabolism in vitro, eugenol (1 mmol/l) was incubated with rat liver and lung microsomes in the presence of an NADPH-regenerating system. Two

of three metabolites detected were identified as GSH conjugates[2]. The third metabolite was also presumed to be a GSH conjugate, but its structure was not deduced. The amount of GSH conjugates formed increased linearly with increasing microsomal protein concentrations up to 1 mg/ml. After 90 min of incubation with 1 mg/ml of protein, about 2.8% eugenol was isolated as GSH conjugates. Omission of the NADPH-generation system or use of heat-inactivated microsomes completely inhibited the formation of GSH conjugates, which suggests that the formation of GSH conjugates is enzyme-mediated. Addition of the CYP450 inhibitors metyrapone, SKF525-A, piperonyl butoxide or β-naphthoflavone inhibited the formation of GSH conjugates by 73–88%. Furthermore, the reaction was shown to be O_2-dependent, as it did not proceed in the presence of *N*2 (i.e. 92% inhibition of conjugate formation). Pre-treatment of rats with the CYP450 enzyme inducer 3-methylcholanthrene increased the rate of liver microsome formation of GSH conjugates (0.66 ± 0.05 versus 1.34 ± 0.06 nmol/min per mg). Incubation of eugenol with rat lung or liver microsomes resulted in the generation of reactive intermediates, as indicated by increased protein binding; however, addition of GSH to the incubation mixture inhibited the binding of eugenol to protein. Conjugation of GSH with this reactive intermediate was not affected by either GSH concentration or the presence of exogenous GSH transferase, suggesting that the conjugation reaction is not enzyme-mediated. Oxidation of 0.5 mmol/l eugenol in the presence of cumene hydroperoxide (added to prevent NADPH interference) resulted in increased absorbance of ultra-violet radiation at 350 nm, which, according to the authors, corresponds to the formation of a eugenol quinone methide intermediate (Thompson et al., 1990).

In other studies of the CYP450 oxidation–GSH conjugation pathway (see Figure 3), concentration- and time-dependent indicators of cytotoxicity were reported when freshly isolated rat hepatocytes were incubated with 0, 0.5, 1 or 1.5 mmol/l of eugenol. At each concentration tested, onset of cell death was observed after 2 h,

Figure 3. Oxidative metabolism of eugenol



From Thompson et al. (1991)

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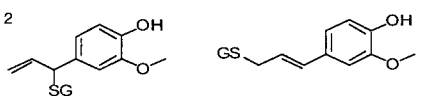
2 

preceded by blebbing of cellular membranes. Cell death was inhibited when 1 mmol/l of eugenol was incubated with rat hepatocytes in the presence of 1 mmol/l of *N*-acetylcysteine. Cellular GSH was depleted by eugenol to less than 30% of control values by 2 h, while control cells showed significant depletion of GSH only after 4 h; until that time, GSH levels were maintained at 90%. Addition of 1 mmol/l of *N*-acetylcysteine prevented the eugenol-induced depletion of GSH. In hepatocytes depleted of GSH by the addition of diethylmaleate, cytotoxicity was observed 2 h before the onset of cytotoxicity in control cells exposed to eugenol only. Covalent binding of radiolabelled eugenol to cellular protein occurred at a linear rate up to 3 h after incubation; however, addition of *N*-acetylcysteine inhibited covalent binding for up to 3 h. Thereafter, *N*-acetylcysteine was depleted, allowing covalent binding to occur. The three metabolites isolated after 5 h of incubation of 1 mmol/l of eugenol with rat hepatocytes were identified as the glucuronic acid, GSH and sulfate conjugates, the glucuronic acid conjugate predominating (i.e. 200 nmol of eugenol glucuronide formed and 25 nmol of each of the other two conjugates) (Thompson et al., 1991).

To study the effect of eugenol on drug-metabolizing enzymes such as CYP450 and UDPGT, male Wistar rats were given 250, 500 or 1000 mg/kg bw per day of eugenol in corn oil by gavage for 10 days. The animals were necropsied 24 h after the last dose, their livers were excised, blood samples were collected and liver microsomes and cytosolic fractions were isolated. No statistically significant changes in body weight, relative liver weight, haematological indices, plasma ALT or aspartate aminotransferase activities or total liver CYP450 content were seen in comparison with controls. Eugenol induced dose-dependent increases in 7-ethoxyresorufin *O*-deethylation and 7-pentoxyresorufin *O*-dependentylation activities, which became statistically significant at 1000 mg/kg bw per day (i.e. 2.5 and 3.4 times that of controls, respectively); these increases are indicative of CYP1A and CYP2B induction. Increased UDPGT activity towards 4-hydroxybiphenyl was observed in rats treated with 500 or 1000 mg/kg bw per day of eugenol (2.3- and 3.2-fold, respectively). Additionally, UDPGT activity towards 4-chlorophenol was significantly increased (1.6- to 3.1-fold) in all rats treated with eugenol. In the liver cytosol of rats treated with eugenol, a dose-dependent increase in GSH transferase activity was reported, which became statistically significant at 500 mg/kg bw per day (i.e. 1.3- and 1.5-fold induction at 500 and 1000 mg/kg bw per day, respectively). The authors proposed that the main metabolic pathway induced by eugenol involves phase-II electrophile processing enzymes (Rompelberg et al., 1993).

When male Wistar rats were given an oral dose of 500 or 1000 mg/kg bw per day of eugenol in corn oil for 10 days, minimal but significant increases (1.2- and 1.3-fold, respectively) in GSH transferase activity towards 1-chloro-2,4-dinitrobenzene ($p < 0.01$) were reported in comparison with vehicle controls. The total CYP450 content of liver microsomes of rats treated with eugenol was comparable to that of controls; however, testosterone hydroxylation at the 16β site, but not at the 16α site, was significantly enhanced at 1000 mg/kg bw per day. Both sites reflect induction of CYP2B1 activity. No significant alterations in testosterone hydroxylation reactions were detected at the 6β, 7α and 2α sites, which reflect activities of CYP3A, CYP1A1 and CYP2C11, respectively. The authors concluded that, while eugenol does not effectively induce CYP450 enzyme activity, it can induce phase-II biotransformation enzymes (Rompelberg et al., 1996a).

The potential of eugenol to be oxidized to a reactive intermediate was investigated in a series of studies in vitro. In one experiment, 500 μmol/l of eugenol were incubated with hydrogen peroxide and myeloperoxidase isolated from human polymorphonuclear leukocytes. Spectral evidence indicated the formation of a quinone methide metabolite in an enzyme-dependent manner. Formation of this metabolite was completely inhibited when reduced GSH (10–50 μmol/l) was present in the reaction mixture at the beginning of the reaction; however, when GSH (50 μmol/l) was added to the reaction mixture after formation of the metabolite had begun, the metabolite reacted directly with the GSH. In myeloperoxidase-catalysed oxidation reactions of eugenol with GSH added at the start of the reaction, most of the added GSH (500 nmol) was oxidized to GSH disulfide (447 ± 33 nmol), 1.6 ± 0.1 nmol conjugating with the quinone methide derivative and 65 ± 4 nmol remaining unchanged. The authors proposed that eugenol forms a phenoxy radical under these conditions, which is reduced back to eugenol through the formation of oxidized GSH (i.e. GSH disulfide) (Thompson et al., 1989).

Eugenol was also shown to inhibit processes associated with oxidative bursts in polymorphonuclear leukocytes stimulated by phorbol myristate acetate, a phorbol ester. Normally, stimulated cells would show a burst in oxygen consumption, ultimately resulting in superoxide and hydrogen peroxide formation. Eugenol (10–1000 μmol/l) inhibited hydrogen peroxide formation in polymorphonuclear leukocytes in a concentration-dependent manner, with 50% inhibition at 100 μmol/l. Furthermore, eugenol was associated with cytotoxicity, as indicated by lactate dehydrogenase leakage and cell death of 50% of polymorphonuclear leukocytes stimulated by phorbol myristate acetate as opposed to 23% of unstimulated cells. The authors concluded that, at concentrations < 100 μmol/l, eugenol forms a covalently bound product and reactive intermediate that depletes GSH. Although no polymorphonuclear leukocyte cytotoxicity was observed at the lower concentrations of eugenol, eugenol did slightly inhibit the cellular oxidative burst leading to the formation of superoxide and hydrogen peroxide (Thompson et al., 1989).

In more recent studies, the quinone methide intermediate, 2-methoxy-4-allylidene-2,5-cyclohexadienone, was synthesized and shown to have a short half-life ($t_{1/2}$ = 613 s or ~ 10 min) in water (Bolton et al., 1995). Subsequently, the cytotoxicity of eugenol and its quinone methide intermediate was evaluated in cloned rat liver cells, which consist of an immortalized cell line containing active CYP450 and normal GSH levels (Thompson et al., 1998). Incubation of cells with eugenol (1, 10, 100 or 1000 μmol/l) resulted in significant depletion of GSH as early as 3 min with 10 μmol/l of eugenol, suppression of gap junction-mediated intercellular communication by 27% and 88% at 10 and 100 μmol/l of eugenol, respectively, within 5 min and inhibition of basal cellular reactive oxygen species generation at eugenol concentrations as low as 10 μmol/l (25% and 75% at 10 and 100 μmol/l of eugenol, respectively). No significant changes were observed at the lowest concentration of eugenol tested, 1 μmol/l. When the quinone methide intermediate was substituted for eugenol, similar decreases in GSH levels and intercellular communication were reported at 1 μmol/l. In contrast to eugenol, the intermediate stimulated the formation of reactive oxygen species 3 min after the start of incubation at a concentration of 10 μmol/l, suggesting that cellular GSH was sufficiently depleted to allow the formation of reactive oxygen species. While 100 μmol/l of eugenol caused no significant increase in cell death, > 90% cell death was observed when liver cells were incubated with a higher concentration of eugenol (1000 μmol/l) or with the

quinone methide intermediate (100 μmol/l) for 2 h. These results support the conclusion that the quinone methide intermediate is the reactive, cytotoxic metabolite. Addition of the ethyl ester of GSH to either incubation mixture completely inhibited the cytotoxic effects of both substances.

In an investigation of the mechanism of the oral antiseptic activity of eugenol, the substance was incubated with cultured male guinea-pig neutrophils. A concentration-dependent increase in superoxide (O^{2-}) was reported at concentrations up to 2 mmol/l of eugenol, accompanied by only a sight increase in cytotoxicity. Maximum superoxide levels were found at 5 mmol/l eugenol, and the value remained constant thereafter; however, most of the cells showed signs of cytotoxicity, and, after 30 s, cell viability was < 50% (Suzuki et al., 1985).

In conclusion, eugenol may participate, to a minor extent, in an oxidation pathway leading to a quinone methide intermediate; however, at the concentrations of the quinone methide intermediate present in liver, effective detoxication by GSH conjugation is expected. In addition, the extensive conjugation of eugenol greatly limits formation of this quinone methide intermediate.

Epoxidation of the allyl moiety

Eugenol is partly (10%) metabolized to an epoxide, which undergoes hydrolysis catalysed by epoxide hydrolase. Hydrolysis yields a diol that can be either conjugated and excreted or oxidized to a lactic acid derivative and then excreted. When eugenol was incubated with rat epithelial cells or rat liver microsomes, 2′,3′-eugenol epoxide was formed (Delaforge et al., 1978). After treatment of adult male Wistar rats with a single intraperitoneal dose of 200 mg/kg bw of eugenol in corn oil, eugenol epoxide, the corresponding diol (dihydrodihydroxy eugenol), allylcatechol epoxide and dihydrodihydroxy allylcatechol were identified after 24 h in urine collected every 2 h (Delaforge et al., 1980). The 24-h liver homogenates obtained from the same rats also contained eugenol epoxide and the corresponding diol. When liver microsomes obtained from rats pre-treated with phenobarbital (80 mg/kg bw; intraperitoneally) for 3 days were incubated with 1 μmol (164 μg) of eugenol, the resulting metabolites were identified as eugenol epoxide, the corresponding diol, allylcatechol epoxide[3] and dihydrodihydroxy allylcatechol. In contrast, cultured adult rat liver cells incubated with 1 mg of eugenol formed only eugenol epoxide and dihydrodihydroxy eugenol and not allylcatechol derivatives.

Eugenol epoxide is presumed to be rapidly detoxicated by the formation of eugenol-2′,3′-diol by epoxide hydrolase, as the epoxide was not detected at any appreciable concentration in vivo (Luo et al., 1992; Guenthner & Luo, 2001).

(d) Other biochemical studies

In a study to investigate the effect of eugenol on smooth muscle activity in the intestines, groups of 8–12 male Wistar-Nossan rats were given 0, 25, 50, 100 or 200 mg/kg bw of eugenol 1 h before administration of 1 ml of an aqueous suspension of 10% charcoal and 5% acacia gum. The small intestine was removed 20 min later, and the distance that the charcoal had travelled from the pylorus was measured. A

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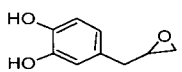
[3] 

significant dose-dependent decrease in the total length of the small intestine travelled by the charcoal suspension was observed, indicating a decrease in smooth muscle action. When 100 mg/kg bw of eugenol were administered 30, 60, 120, 180 or 240 min before administration of the charcoal suspension, maximum transit distance was observed after 60 min. The authors suggested that eugenol at the doses used in this study might inhibit the spontaneous activity of the longitudinal gut muscle, possibly by inhibiting prostaglandin synthesis (Bennett et al., 1988).

To investigate the potential of eugenol to inhibit prostaglandin synthesis, homogenized human colon mucosa was incubated with 0, 1, 10 or 100 μg/ml of eugenol. Concentration-dependent inhibition of prostanoid formation was found, with statistically significant inhibition at concentrations of 10 and 100 μg/ml and as low as 1 μg/ml for inhibition of thromboxane B_2. Human polymorphonuclear leukocytes incubated with ^{14}C-arachidonic acid in the presence of 0, 1, 10 or 100 μg/ml of eugenol showed marked inhibition (approximately 85%) in the formation of 5-hydroxy-eicosatetraenoic acid at the highest concentration tested (100 μg/ml) (Bennett et al., 1988).

The potential effect of eugenol as a muscle relaxant was studied in vitro in longitudinal strips of isolated human gastric and colon muscle and Wistar-Nossan rat forestomach muscle. In human tissues, eugenol at a concentration of 300 ng/ml reduced the spontaneous contractility of the muscle strips. Furthermore, eugenol at 0.2–100 μg/ml decreased the tone of the muscle strips from either segment of the gastrointestinal tract; however, in circular muscle, eugenol (0.22–0.88 μg/ml) increased the tone but induced either no change (8–200 μg/ml) or a decrease (0.2–11 μg/ml) in the tone of the colon muscle. Eugenol also inhibited acetylcholine-induced contraction in the colon muscles, although its effect was more variable in the stomach muscles. Strips of rat forestomach muscle were suspended in a bath of Krebs solution with 1, 5, 10 or 50 μg/ml of eugenol for 2 min before addition of 2.5 ng/ml each of prostaglandin E2, 5-hydroxytryptamine or acetylcholine. Spontaneous muscle contractions were reduced in a dose-dependent manner in eugenol-treated samples when compared with controls. Rat uterine muscle bathed with eugenol (1–50 μg/ml) showed a concentration-dependent reduction in bradykinin-induced contractions (7–95%, $p < 0.05$ to < 0.001). Similarly, rabbit jejunum muscle showed a concentration-dependent decrease of 22–90% ($p < 0.05$ to < 0.005) in spontaneous muscle contractions when bathed in eugenol (2.5–50 μg/ml). In two human myometrium specimens, eugenol (22 μg/ml) reduced the tone and contractions in response to prostaglandin $F_{2\alpha}$ (Bennett et al., 1988).

Prostaglandin E2 is known to stimulate intestinal fluid accumulation. When inbred Lewis-Nossan rats were given 10–100 mg/kg bw of eugenol by gavage 4 h before receiving 2 mg/kg bw of prostaglandin E2, dose-dependent ($p < 0.05$ to < 0.001) inhibition of intestinal fluid accumulation was observed when compared with controls. Rat paw oedema induced by carrageenan was reduced in a dose-dependent manner when the rats were given 100–500 mg/kg bw of eugenol 1 h before treatment with carrageenan. At doses of 10 or 50 mg/kg bw of eugenol, no reduction was observed. These results indicate that, in rats, high oral doses of eugenol can inhibit prostaglandin synthesis and have an anti-diarrhoeal effect (Bennett et al., 1988).

2.3.2 *Toxicological studies*

The toxicological studies are summarized below according to duration, flavouring agent and then species. In order to preserve the continuity of the studies performed within the National Toxicology Program, however, the short-term studies of toxicity and the carcinogenicity studies are both discussed in the section on long-term studies in the sequence in which they were conducted.

(a) Acute toxicity

Oral LD_{50} values have been reported for three of the seven substances in this group (Table 4). In rats, the LD_{50} values ranged from 1194 mg/kg bw for eugenol to 3400 mg/kg bw for eugenyl formate. The values for eugenol were 3000 mg/kg bw in mice and 2130 mg/kg bw in guinea-pigs. These results indicate that eugenol and related hydroxyallylbenzene derivatives given orally have little acute toxicity (Sober et al., 1950; Jenner et al., 1964; Hagan et al., 1965; Bär & Griepentrog, 1967; Gruebner et al., 1972; Moreno, 1972; Beroza et al., 1975; Moreno, 1977).

(b) Short-term studies of toxicity

The results of short-term and long-term studies of toxicity and studies of carcinogenicity with eugenol and related allylhydroxybenzene derivatives are summarized in Table 5.

Table 4. Results of studies of acute toxicity with orally administered eugenol and related hydroxyallylbenzene derivatives

No.	Flavouring agent	Species; sex	LD_{50} (mg/kg bw)	Reference
1529	Eugenol	Rat; M, F	2680	Jenner et al. (1964)
1529	Eugenol	Mouse; NR	3000	Jenner et al. (1964)
1529	Eugenol	Rat; M, F	1194[a]	Beroza et al. (1975)
1529	Eugenol	Guinea-pig; M, F	2130	Jenner et al. (1964)
1529	Eugenol	Rat; NR	2680	Bär & Griepentrog (1967)
1529	Eugenol	Rat; M, F	2680	Hagan et al. (1965)
1529	Eugenol	Guinea-pig; M, F	2130	Hagan et al. (1965)
1529	Eugenol	Mouse; M, F	3000	Hagan et al. (1965)
1529	Eugenol	Rat; M	1930	Gruebner et al. (1972)
1529	Eugenol	Rat; NR	1930	Sober et al. (1950)
1530	Eugenol formate	Rat; NR	3400	Moreno (1977)
1530	Eugenol formate	Rat; M	2600	Moreno (1972)
1530	Eugenol formate	Rat; M, F	1670	Jenner et al. (1964)
1531	Eugenol acetate	Rat; M, F	1670	Bär & Griepentrog (1967)

M, male; F, female; NR, not reported

[a] LD_{50} value of 3980 mg/kg bw was reported for a mixture of 7:3 phenethyl propionate:eugenol.

Table 5. Results of short-term studies of toxicity and long-term studies of toxicity and carcinogenicity on eugenol and related hydroxyallylbenzene derivatives

No.	Substance	Species; sex	No. test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL mg/kg bw per day)	Reference
Short-term studies of toxicity							
1529	Eugenol	Rat; M	1/5	Diet	28	2000[c]	Hirose et al. (1987)
1529	Eugenol	Rat; M	1/20	Gavage	34	< 1400[d]	Hagan et al. (1965, 1967)
1529	Eugenol	Rat; M, F	1/20	Diet[e]	90	M: 84.1[c] F: 94.4[c]	Trubek Laboratories Inc. (1958)
1529	Eugenol	Rat; NR	NR	Diet	133	1000[c]	Bär & Griepentrog (1967)
1529	Eugenol	Rat; M, F	2/20	Diet	133	1000[c]	Hagan et al. (1967)
1531	Eugenyl acetate	Rat; M, F	3/20	Diet	133	1000[c]	Hagan et al. (1967)
1531	Eugenyl acetate	Rat; NR	NR	Diet	133	1000[c]	Bär & Griepentrog (1967)
1529	Eugenol	Mouse; M, F	1/114	Gavage	35[f]	410[c]	Miller et al. (1983)
1529	Eugenol	Mouse; M, F	5/20	Diet	91	900[c]	National Toxicology Program (1983)
1529	Eugenol	Rat; M, F	5/20	Diet	91	1250[c]	National Toxicology Program (1983)
1529	Eugenol	Mouse; F	2[g]/30	Diet	365	750[c]	Miller et al. (1983)

Table 5 (contd)

No.	Substance	Species; sex	No. test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL mg/kg bw per day)	Reference
Long-term studies of toxicity and carcinogenicity							
1529	Eugenol	Mouse; M, F	2/100	Diet	735	450	National Toxicology Program (1983)
1529	Eugenol	Rat; M, F	3/50-100[h]	Diet	735	M: 300[c] F: 625c	National Toxicology Program (1983)

M, male; F, female; NR, not reported

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] Study performed with either a single or multiple doses that had no adverse effect. The value is therefore not a true NOEL, but is the highest dose tested that had no adverse effects. The actual NOEL might be higher.

[d] Rats treated with an initial dose of 1400 mg/kg bw of eugenol, which was increased gradually to 4000 mg/kg bw over the course of the 34-day study

[e] The test material was a mixture of flavour compounds, consisting of eugenol (123 ppm), anisic aldehyde (10 ppm) and heliotropine (22 ppm), which were blended in proportion to their reported levels of use and incorporated into the test diet at a level providing a daily dose of 100 mg/kg bw of the flavour mixture. Actual mean intakes of the flavour mixture were 106 and 119 mg/kg bw per day for male and female rats, respectively. On the basis of the concentration at which eugenol was incorporated in the test diet (79.4%), the mean daily intakes of the flavour mixture corresponded to average daily intakes of approximately 84.1 and 94.4 mg/kg bw of eugenol by male and female rats, respectively.

[f] Mice treated twice weekly with 410 mg/kg bw of eugenol for a total of 10 doses and autopsied at end of the 14-month study

[g] In addition to the eugenol-supplemented diet, one group also received 0.5% phenobarbital in drinking-water for the duration of the study.

[h] Total of three test groups in the study but only two groups per sex. Male rats were given diets providing 150 or 300 mg/kg bw per day and female rats were given diets providing 300 or 625 mg/kg bw per day of eugenol.

Eugenol (No. 1529)

Rats

Groups of three male Wistar rats were given 0, 250, 500 or 1000 mg/kg bw per day of eugenol in corn oil by gavage for 10 days. The animals were observed for clinical signs of toxicity, and body weights were measured on days 0, 6 and 10 of the study. A statistically nonsignificant but dose-dependent decrease in body-weight gain (by about 6%) was reported in animals given eugenol when compared with controls. Eugenol had no effect on haematological or clinical chemistry parameters, and the relative liver weights of eugenol-treated rats were comparable to those of controls (Rompelberg et al., 1993).

In a 28-day study, groups of five 6-week old male Fischer 344 rats were maintained on a diet designed to provide 0 or 2% eugenol, corresponding to an estimated daily intake of 2000 mg/kg bw (Food & Drug Administration, 1993). The rats were given free access to food and water and were weighed weekly. They were killed for autopsy at the end of week 4, when the livers were weighed and the stomachs were preserved in buffered formalin; the posterior and anterior walls of the forestomach were cut into strips and examined histologically. Body-weight gain was depressed by 10–15% in rats given eugenol as compared with controls; however, the relative liver weights were not significantly different between the two groups. No gross or histological changes were found in the forestomach (Hirose et al., 1987).

Twenty male weanling Osborne-Mendel rats were given 1400 mg/kg bw per day of eugenol by stomach tube, and the dose was gradually increased to 4000 mg/kg bw per day during the 34-day study. A few deaths were reported at 2000 mg/kg bw per day, and the number increased with increasing doses; eight animals survived to 34 days, while 15 animals survived long enough to receive the dose of 4000 mg/kg bw per day. Weekly measurements of body weight, general condition and food intake showed no differences between treated and control rats. All surviving animals were killed, and haematological examinations (white and red cell counts, haemoglobin concentration and erythrocyte volume fraction) were performed. At necropsy, liver, kidneys, spleen, heart and testes were weighed. These organs, the abdominal and thoracic viscera and one hind leg were preserved in buffered formalin–saline solution. Eugenol caused slight adrenal enlargement, with marked yellow discolouration, and slight enlargement of the liver, which was found microscopically to be due to slight liver-cell enlargement. Macroscopic examination of the forestomach showed that the mucosa contained coalescent areas covered with a thick, flaky, white material punctuated with minute ulcers. Microscopic examination of the forestomach showed moderately severe hyperplasia and hyperkeratosis of the stratified squamous epithelium associated with focal ulceration. Mild osteoporosis was also found (Hagan et al., 1965, 1967).

Groups of 10 weanling Osborne-Mendel rats of each sex were given diets containing 0, 1000 or 10 000 ppm per day of eugenol for 19 weeks, corresponding to estimated daily intakes of 0, 50 and 500 mg/kg bw, respectively (Food & Drug Administration, 1993). Weekly measurements of body weight, general condition and food intake showed no differences between test and control rats. All animals were killed, and haematological examinations (white and red cell counts, haemoglobin concentration and erythrocyte volume fraction) were performed. At necropsy, liver, kidneys, spleen, heart and testes were weighed. These organs, the abdominal and

thoracic viscera and one hind leg were preserved in buffered formalin–saline solution. No effects were observed that were attributable to treatment (Hagan et al., 1967).

In a 90-day study, 10 male and 10 female rats of unspecified strain were fed a test diet designed to provide a 100 mg/kg bw of a flavour mixture consisting of eugenol, anisic aldehyde and heliotropine, which were blended at concentrations proportional to their reported levels of use: eugenol (No. 1529), 123 ppm; anisic aldehyde, 10 ppm; and heliotropine, 22 ppm. The actual mean intakes of the mixture were reported to be 106 and 119 mg/kg bw per day for male and female rats, respectively. On the basis of the concentration at which eugenol was incorporated in the flavour mixture (about 79.4%), the mean daily intake of the flavour mixture corresponded to average daily intakes of about 84.1 and 94.4 mg/kg bw of eugenol for male and female rats, respectively. Control rats were fed unsupplemented diets for the duration of the study. All the rats were observed weekly for growth and food intake. After 12 weeks of feeding, the urine of three rats of each sex per group was examined for glucose and albumin concentrations, and blood haemoglobin levels were determined. At the end of the study, gross autopsies were performed on all survivors, the liver and kidneys were weighed, and tissues were preserved for histological examination. The appearance and behaviour of the treated rats were comparable to those of controls, and all animals survived to the end of the study. The net body-weight gain and food use efficiency were lower for test animals than for controls, but these differences did not reach statistical significance. At 12 weeks, analysis of the urine of male rats receiving the test mixture revealed the presence of albumin, which was not observed in treated females. According to the authors, this is a common observation in male rats and might be attributable to the presence of semen in the urine. Gross examination at autopsy revealed occasional respiratory infection in both groups. The liver and kidney weights of both groups were comparable and were within normal limits (Trubek Laboratories Inc., 1958).

Eugenyl acetate (No. 1531)

Rats

An unspecified number and strain of rats were given a diet containing 10 000 ppm eugenyl acetate for 19 weeks, corresponding to an estimated daily intake of 1000 mg/kg bw (Food and Drug Administration, 1993). No adverse effects were reported. No further experimental details were provided (Bär & Griepentrog, 1967).

Groups of 10 weanling Osborne-Mendel rats of each sex were fed diets providing 0, 1000, 2500 or 10 000 ppm per day of eugenyl acetate for 19 weeks, corresponding to estimated daily intakes of 0, 100, 250 and 1000 mg/kg bw, respectively (Food & Drug Administration, 1993). Weekly measurements of body weight, general condition and food intake showed no differences between test and control rats. All animals were Killed, and haematological examinations (white and red cell counts, haemoglobin concentration and erythrocyte volume fraction) were performed. At necropsy, liver, kidneys, spleen, heart and testes were weighed. No adverse effects were observed (Hagan et al., 1967).

(c) Long-term studies of toxicity and carcinogenicity (see Table 5)

Eugenol (No. 1529)

Mice

In a study of the carcinogenic potential of allylalkoxybenzene and allylhydroxybenzene derivatives, two groups of 30 CD-1 female mice (mean weight, 21 g) were maintained on a diet containing 0.5% eugenol, estimated to correspond to an average daily intake of 750 mg/kg bw (Food & Drug Administration, 1993). The diet was continued for 12 months, when the mice were allowed a 6-month recovery period. One of the groups receiving eugenol in the diet also received 0.05% phenobarbital in the drinking-water for the entire 18 months. Two control groups were included: mice that received control diet only, and mice that received control diet and 0.05% phenobarbital in their drinking-water. Survival at 18 months was comparable between eugenol-fed animals with and without phenobarbital treatment and control animals. The body-weight gains of treated mice, measured at 1, 4 and 8 months, were comparable to those of controls. One pulmonary adenoma, two thymic lymphomas and one mammary adenoacanthoma were found in animals given eugenol without phenobarbital, while control mice without phenobarbital treatment had one pulmonary adenoma. Phenobarbital-treated controls also had one case each of pulmonary adenoma and liver haemangioendotheliosarcoma. The authors concluded that no adverse effects could be attributed to treatment (Miller et al., 1983).

In another segment of the same study, 59 male and 55 female CD-1 mice were given 2.5 μmol/g bw of eugenol (about 410 mg/kg bw) by gavage twice a week for 10 doses, beginning at 4 days of age. The mice were weaned at 35 days of age, and hepatomas were evaluated at 14 months. The incidence of hepatomas was essentially the same in the test and control groups: 25% in eugenol-treated males (0.5 hepatomas/mouse) and 24% in vehicle control males (0.6 hepatomas/mouse). The incidence of hepatomas in eugenol-treated females (none) was not statistically significantly different from that in vehicle control females (2%, 0.02 hepatomas/mouse) (Miller et al., 1983).

A group of 52 male CD-1 mice were given a total dose of 9.45 μmol/mouse of eugenol or eugenol 2′,3′-oxide by intraperitoneal injection, distributed in a ratio of 1:2:4:8, on days 1, 8, 15 and 22 of life. These doses correspond to 0.63, 1.26, 2.52 and 5.04 μmol/mouse or 73.9, 59.1, 59.1 and 63.7 mg/kg bw, respectively. The mice were weaned at 22 days of age. At 12 months, hepatomas were recorded in 24% of mice receiving eugenol and 31% of mice receiving eugenol 2′,3′-oxide (0.6 and 0.5 hepatomas/mouse), with 26% in vehicle controls (0.5 hepatomas/mouse). These differences were not statistically significant (Miller et al., 1983).

In a 14-day study, groups of five $B6C3F_1$ mice of each sex were given diets containing 6000, 12 500, 25 000, 50 000 or 100 000 ppm of eugenol on 5 days/week for a total of 10 doses, corresponding to estimated daily intakes of 900, 1875, 3750, 7500 and 15 000 mg/kg bw, respectively (Food & Drug Administration, 1993). The animals were observed twice daily, and body weights and clinical findings were recorded at the start, on day 8 and at the end of the study. All mice at 100 000 ppm and 60% of male mice at 50 000 ppm died. A dose-related decrease in mean body-weight gain was observed in male mice at doses ≥ 12 500 ppm and in female mice at doses ≥ 25 000 ppm (National Toxicology Program, 1983).

Groups of 10 B6C3F$_1$ mice of each sex were given diets containing 0, 400, 800, 1500, 3000 or 6000 ppm of eugenol on 5 days/week for 13 weeks, corresponding to estimated daily intakes of 0, 60, 120, 225, 450 and 900 mg/kg bw, respectively (Food & Drug Administration, 1993). The animals were observed twice daily for clinical signs or deaths, and body weights were recorded weekly. At the end of the 91-day study, necropsies were performed on all animals, and complete histopathological examinations were conducted on mice at the highest dose and on the controls. No deaths occurred, and no compound-related effects were reported on body weight or on gross or histopathological appearance at any dose (National Toxicology Program, 1983).

Groups of 50 B6C3F$_1$ mice of each sex were given diets containing 0, 3000 or 6000 ppm of eugenol for 105 weeks, corresponding to estimated daily intakes of 0, 450 and 900 mg/kg bw, respectively (Food & Drug Administration, 1993). The animals were observed twice daily for morbidity or mortality, and clinical findings were recorded monthly. The mice were weighed weekly for the first 13 weeks, then monthly to week 93 of the study and every 2 weeks thereafter. Necropsies were performed on all animals at the end of the study. All major tissues, organs and visible lesions were examined grossly, microscopically and macroscopically. No compound-related clinical signs were observed in any of the mice during the study. The survival and mean body weights of all treated animals were similar to those of controls throughout the study, with the exception of females at 6000 ppm, which had mean body weights that were 14% and 11% lower than those of controls in weeks 101 and 104, respectively. The average daily feed consumption by mice at 3000 ppm and 6000 ppm was 97% and 94% that of controls for males, and 95% and 90% for females, respectively. Five males at 3000 ppm were accidentally killed at week 13 and were subsequently excluded from the statistical analyses. The incidence of hepatocellular adenomas and carcinomas among males at the lower dose was significantly increased ($p < 0.05$); but males at the higher dose showed no increase. When the incidences of liver adenomas and carcinomas were combined, a slight increase was found in males at the higher dose (18/49, 37%) as compared with controls (14/50, 28%), but the difference was not statistically significant. The incidences of hepatocellular adenomas and carcinomas were not significantly increased in female mice; however, the combined incidence of hepatocellular adenomas and carcinomas showed a positive dose-related trend: controls, 2/50 (4%); lower dose, 7/49 (14%); higher dose, 9/49 (18%). One male at the lower dose had a tumour that was described as having some areas characteristic of hepatocellular carcinoma and areas of disorderly proliferation of structures resembling bile ducts. The authors classified the tumour as a mixed hepatocellular–cholangiocarcinoma. The incidence of metastasized tumours of the lung was similar in test and control groups of male mice (control, 2; lower dose, 3; higher dose, 2). One tumour in a female at the lower dose had metastasized. The incidence of follicular-cell adenomas of the thyroid gland in male mice showed a significantly ($p < 0.05$) increased trend: control, 0/48 (0%); lower dose, 0/49 (0%); higher dose, 3/49 (6%). The corresponding rates in treated females were not significantly different from those in males: control, 2/48 (8%); lower dose, 0/47 (0%); higher dose, 1/49 (2%). The authors concluded that, under the conditions of this study, there was equivocal evidence for carcinogenicity, as eugenol increased the incidences of both carcinomas and adenomas of the liver in male mice at 3000 ppm and increased the combined incidence of hepatocellular carcinomas and adenomas in female mice (Table 6; National Toxicology Program, 1983).

Table 6. Incidences of hepatocellular neoplasms associated with dietary administration of eugenol to mice for 2 years

Sex	Neoplasm	Control	3000 ppm	6000 ppm
Male	Adenoma	4/50 (8%)	13/50 (26%)*	10/49 (20%)
	Carcinoma	10/50 (20%)	20/50 (40%)*	9/49 (18%)
Female	Adenoma or carcinoma	14/50 (28%)	28/50 (56%)**	18/49 (37%)
	Adenoma	0/50 (0%)	4/49 (8%)	3/49 (6%)
	Carcinoma	2/50 (4%)	3/49 (6%)	6/49 (12%)
	Adenoma or carcinoma	2/50 (4%)	7/49 (14%)	9/49 (18%)*

From National Toxicology Program (1983). Incidences of liver adenomas or carcinomas in control B6C3F$_1$ mice in National Toxicology Program carcinogenicity (feeding) studies: males, 42.2% (10–68%); females, 23.6% (6–56%) (Haseman et al., 1998)

* Significantly different ($p < 0.05$) from the control group by the Fisher exact test

** Significantly different ($p < 0.01$) from the control group by the Fisher exact test

Liver adenomas and carcinomas are common neoplasms in male B6C3F$_1$ mice in National Toxicology Program studies. In controls in dietary studies, the background incidence of hepatocellular tumours was 42.2% in male mice and 23.6% in female mice (Haseman et al., 1998). The high incidences of hepatocellular adenomas and carcinomas in both control (28%) and treated (lower dose, 56%; higher dose, 37%) male mice in comparison with female mice (control, 4%; lower dose, 14%; higher dose, 18%) clearly demonstrates the sensitivity of male B6C3F$_1$ mouse liver to neoplastic changes. As the combined incidence of hepatocellular tumours in male mice showed no dose–response relation and the incidence of tumours in mice at the higher dose was not significantly different from that of the control group, the appearance of liver tumours in male mice in this study was considered to have been spontaneous and unrelated to administration of the test substance. Although the incidence of hepatocellular adenomas and carcinomas in treated female mice was higher than in the control group, the response was not statistically significant and, in the case of adenomas, was not dose-related. The combined incidence of hepatic tumours in control and treated females showed a dose-related trend (control, 4%; lower dose, 14%; higher dose, 18%); however, the difference in incidence between the controls and females at the lower dose was not statistically significant. According to the National Toxicology Program, the profile of neoplastic responses in both male and female mice in 2-year bioassays are consistent with the high background levels of hepatocellular neoplasms in this species and strain (Maronpot et al., 1987).

A second factor in explaining the results of the National Toxicology Program (1983) study might be the location of housing during the 2-year feeding period (Young, 1987). It is common practice in the conduct of long-term studies to cage mice together and to locate cages of mice receiving the same treatment contiguously, rather than randomly. This practice was observed during this study. Nevertheless, it was postulated that local room effects might have been associated with the occurrence of hepatic tumours, particularly in male mice at the lower dose. In a cage-by-cage analysis, the incidence of hepatic lesions in male mice in cages 1–5 was found to be higher (80%) than that of hepatic tumours in the rest of the mice in the study (32%). Furthermore, on the basis of 43 National Toxicology Program studies in mice reviewed by Haseman et al. (1984), the incidence of hepatic carcinoma in control groups was

≤ 36%, whereas the incidence of hepatic carcinoma in cages 1–5 housing male mice at the lower dose was 64%. Young (1987) concluded that, as the cages were located systematically and as the increase in proliferative hepatic lesions, primarily carcinoma, was concentrated in the first 5 of 10 contiguous cages housing mice at the lower dose, the effect might have been due to room location and not eugenol.

In view of these observations, the hepatic neoplasms in the National Toxicology Program bioassay are not relevant to the safety of eugenol in humans, who have a low intake from use of the compound as a flavour ingredient. This conclusion is based on: the high incidence of spontaneous hepatocellular neoplasms (adenomas and carcinomas) in $B6C3F_1$ mice; the absence of consistent dose–response relations; the effect of the location of animal housing on tumour incidence; the lack of hepatocellular neoplastic effects in a parallel study in rats (discussed below); and the relatively high dietary levels (450 and 900 mg/kg bw) tested, which are at least > 8000 times the estimated daily intake of eugenol from its use as a flavour ingredient (18 μg/kg bw per day in Europe and 56 μg/kg bw per day in the USA) (see Table 2).

Rats

When 20 male Fischer rats were each given a total dose of 2 mmol (about 1029 mg/kg bw) of eugenol-2′,3′-oxide by subcutaneous injection twice weekly for a total of 20 injections, two sarcomas were reported at the injection site and one keratoacanthoma, one sebaceous gland carcinoma and one fibromyosarcoma; however, no hepatic carcinomas were reported. In comparison, control rats that received only the vehicle (trioctanoin) had no sarcomas at the injection site or hepatic carcinomas but had one leiomyosarcoma of the abdominal cavity, one subcutaneous fibrosarcoma and one pulmonary adenoma (Miller et al., 1983).

In a 14-day study, groups of five Fischer 344/N rats of each sex were given diets containing 6000, 12 500, 25 000, 50 000 or 100 000 ppm eugenol on 5 days/week for a total of 10 doses, corresponding to estimated daily intakes of 600, 1250, 2500, 5000 and 10 000 mg/kg bw, respectively (Food & Drug Administration, 1993). The animals were observed twice daily, and body weights and clinical findings were recorded at the start, on day 8 and at the end of the study. At 100 000 ppm, 20% of male and 100% of female rats died. A dose-related decrease in mean body-weight gain was observed for males and females at doses ≥ 25 000 ppm (National Toxicology Program, 1983).

In a 13-week study, groups of 10 Fischer 344/N rats of each sex (only 9 males at 800 ppm and 9 females at 6000 ppm) were given diets containing 0, 800, 1500, 3000, 6000 or 12 500 ppm eugenol, corresponding to estimated daily intakes of 0, 80, 150, 300, 600 and 1250 mg/kg bw, respectively (Food & Drug Administration, 1993). The animals were observed twice daily for clinical signs and mortality, and body weights were recorded weekly. At termination, necropsies were performed on all animals, and complete histopathological examinations were made on rats at the highest dose and the control group. All animals survived the duration of the study. In comparison with the controls, the final mean body weights were reduced by 10% and 6% in male and female rats at the highest dose, respectively. No compound-related histopathological effects were observed (National Toxicology Program, 1983).

Groups of 50 male Fischer 344/N rats were given diets containing eugenol at a concentration of 3000 or 6000 ppm, while groups of 50 female Fischer 344/N

rats received diets containing 6000 or 12 500 ppm eugenol on 5 days/week for 105 weeks (National Toxicology Program, 1983). These dietary levels correspond to estimated daily intakes of 150, 300 and 625 mg/kg bw, respectively (Food & Drug Administration, 1993). As controls, groups of 40 male and 40 female rats were given basal diet throughout the study. Animals were observed twice daily for mortality, and clinical findings were recorded monthly. The rats were weighed weekly for the first 13 weeks, then monthly to week 93 of the study, and every 2 weeks thereafter. Necropsies were performed on all animals at the end of the study. All major tissues, organs and visible lesions were examined grossly, microscopically and macroscopically. The survival rates of treated males (52% at 3000 ppm; 74% at 6000 ppm) and females (72% at 6000 ppm; 88% at 12 500 ppm) were not significantly different from those of controls (58% of males; 75% of females). The mean body weights of females at 12 500 ppm were lower than those of controls. The average daily feed consumption of females at the higher and lower doses were 94% and 91% that of controls, respectively. The statistical significance of the results was not reported.

The incidence of alveolar or bronchial adenomas or carcinomas of the lung was significantly increased ($p < 0.05$) in males at 3000 ppm [5/49 (10%)] relative to control males (0/49), but not in males at 6000 ppm [2/50 (40%)]. The corresponding rates in females at the lower [1/50 (2%)] and higher doses (0/50) were not significantly different from their controls [1/39 (3%)]. Females at the lower dose [11/49 (22%)], however, had a significantly increased incidence ($p < 0.05$) of C-cell adenomas of the thyroid compared with the controls [3/40 (8%)] and with females at the higher dose [2/50 (4%)]. Conversely, there were statistically significant negative trends ($p < 0.05$) in the incidence of C-cell adenomas [control, 4/40 (10%); lower dose, 5/50 (10%); higher dose, 0/50 (0%)] and in the combined incidence of adenomas and carcinomas of the thyroid [control, 7/40 (28%); lower dose, 8/50 (16%); higher dose, 2/50 (4%)] in treated male rats compared with controls. These results are not unexpected, given the high spontaneous incidence of C-cell thyroid gland tumours in Fischer 344 rats. The background incidences of C-cell adenomas and carcinomas in controls in previous dietary studies were 13% (2–35%) and 1.8% (0–6%), respectively, in male rats and 11.7% (4–22%) and 1.9% (0–4%), respectively, in female rats (Haseman et al., 1998).

Female rats showed a significant positive trend ($p < 0.05$) in the incidence of endometrial stromal polyps or sarcomas of the uterus [control, 6/40 (15%); lower dose, 6/50 (12%); higher dose, 16/50 (32%)], but the incidence was not statistically significant in females at the lower dose and was only marginally increased ($p = 0.051$) in females at the higher dose relative to controls. The incidences of endometrial stromal polyps of the uterus in controls (16%) and females at the higher dose (32%) were higher than the average for the research laboratory in which the study was performed (15%) (National Toxicology Program, 1983) and higher than the average for controls in other dietary studies (14.2%) (Haseman et al., 1998). Female rats also showed a significantly decreased incidence of fibroadenomas of the mammary gland ($p < 0.05$) at the lower [8/50 (16%)] and the higher doses [6/50 (12%)] in comparison with the incidence in control animals [14/40 (35%)]. The authors reported that the control female rats in this study had a higher incidence of mammary gland fibroadenomas than controls in other dietary studies [41.2% (8–60%)] (Haseman et al., 1998) and concluded that there was no evidence of carcinogenicity in male or female rats (National Toxicology Program, 1983).

(d) Genotoxicity

Two of the substances in this group comprising eugenol and hydroxyallylbenzene derivatives have been tested for mutagenicity or genotoxicity. The results are summarized in Table 7 and described below.

In vitro

In standard assays for mutagenicity in *Salmonella typhimurium*, eugenol was not mutagenic in strains TA92, TA94, TA97, TA98, TA100, TA102, TA1530, TA1531, TA1532, TA1535, TA1537, TA1538 and TA1964 at concentrations ≤ 107 000 μg/plate (about 652 μmol/plate), with or without metabolic activation (Dorange et al., 1977; Green & Savage, 1978; Rockwell & Raw, 1979; Swanson et al., 1979; Douglas et al., 1980; Eder et al., 1980; Florin et al., 1980; Nestmann et al., 1980; Rapson et al., 1980; Yoshimura et al., 1981; Eder et al., 1982; Pool & Lin, 1982; Sekizawa & Shibamoto, 1982; To et al., 1982; Haworth et al., 1983; National Toxicology Program, 1983; Ishidate et al., 1984; Orstavik & Hongslo, 1985; Amonkar et al., 1986; Miller et al., 1986; Tennant et al., 1987; Schiestl et al., 1989; Azizan & Blevins, 1995; Sukumaran & Kuttan, 1995). Eugenol was mutagenic in *S. typhimurium* strain TA1535 but only when 3′-phosphoadenosine-5′-phosphosulfate was included in the metabolic activation mix. Eugenol did not show a concentration-dependent increase in mutagenic activity. The authors suggested that the result was an artefact of the experimental design, as mutation was not reported as the number of revertants per survivor (induced mutation frequency) but only as the number of revertants per plate (To et al., 1982).

Eugenol 2′,3′-epoxide was not mutagenic in *S. typhimurium* strains TA1537, TA1538 and TA98 but showed mutagenic potential in strains TA1535 (Dorange et al., 1977; Swanson et al., 1979) and TA100 (Dorange et al., 1977).

In a study designed to investigate the mutagenicity of the metabolites of eugenol, Sprague-Dawley rats were given a single dose of 0.5 ml of eugenol (about 2140 mg/kg bw) by gavage, and their urine was collected for 24 h. Before being administered to rats, samples of 0.05–100 μl (53.5–107 000 μg/plate) were each assayed for reverse mutation potential in *S. typhimurium* strains TA98 and TA100 in the presence of metabolic activation. Negative results were obtained. To assess the genotoxic potential of urinary metabolites, the urine was assayed directly or extracted with ether after dilution in a phosphate buffer and treatment with β-glucuronidase to hydrolyse glucuronide conjugates. The 24-h urine samples (500 μl), ether extracts of the urine and aqueous fractions of the extracts were then incubated separately with *S. typhimurium* strains TA98 and TA100 with S9 activation. The urinary solutions isolated from rats given 0.5 ml of eugenol did not show evidence of mutagenicity in either TA98 or TA100 (Rockwell & Raw, 1979).

No mutagenic potential was reported when 60, 120, 300 or 600 μg/plate (0.37, 0.74, 1.8 or 3.6 μmol/plate) eugenol was incubated with *Escherichia coli* WP2 uvrA (trp^-) (Sekizawa & Shibomoto, 1982). Rec assays in *Bacillus subtilis* M45 (rec^-) and H17 (rec^+) performed with 400 μg/disc (2.4 μmol/disc) in the absence of S9 activation gave a positive result (Sekizawa & Shibamoto, 1982); however, DNA repair assays with ≤ 100 000 μg/disc (609 μmol/disc), under standard incubation conditions, gave uniformly negative results (Oda et al., 1979; Yoshimura et al., 1981). With an inhibition zone difference between rec^- and rec^+ cells of 6.9 mm, Sekizawa and

Table 7. Studies of genotoxicity on eugenol and related allylhydroxybenzene derivatives

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
In vitro						
1529	Eugenol	Reverse mutation	*S. typhimurium* TA1530, TA1531, TA1532, TA1964	0.02 or 0.2 mol/l (3284 or 32 841 µg/ml)[a]	Negative[b]	Green & Savage (1978)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA1530, TA1531, TA1532, TA1964	1–5 mg/plate (1000–5000 µg/plate)	Negative[c]	Green & Savage (1978)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1538, TA1537	2000 µg/plate	Negative[d]	Nestmann et al. (1980)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA92, TA94, TA98, TA100, TA1535, TA1537	≤ 2000 µg/plate	Negative[b,e]	Ishidate et al. (1984)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	3 µmol/plate (492.6 µg/plate)[a]	Negative[d,f]	Florin et al. (1980)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1538, TA1537	32.84 µg/plate	Negative[c,f]	Dorange et al. (1977)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	333 µg/plate	Negative[d,g]	Tennant et al. (1987)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1538, TA1537	60, 120, 300 or 600 µg/plate	Negative[c,h] Negative[b,e,h]	Sekizawa & Shibamoto (1982)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA104	3000 µg/plate	Negative[d,f,i]	Miller et al. (1986)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Not reported	Negative[d,j]	Eder et al. (1982)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0.01–3 µl per 2-ml incubation volume (5–1605 µg/ml)[k]	Negative[d,h,j]	Eder et al. (1980)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA100	0.1–1000 µg/plate	Negative	Rapson et al. (1980)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100	2500 µg/ml	Negative[d]	Orstavik & Hongslo (1985)

Table 7 (contd)

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	10, 150, 300, 600 or 1200 µg/plate	Negative[d,l]	Amonkar et al. (1986)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1538, TA1537	2000 µg/plate	Negative[d]	Douglas et al. (1980)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA100, TA102, TA1535	50–400 µg/plate	Negative[d]	Sukumaran & Kuttan (1995)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100	≤ 30 µmol/plate (4926 µg/plate)[a]	Negative[d,m]	Swanson et al. (1979)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0.5, 5, 50, 500 or 5000 µg/plate	Negative[d,n]	Pool & Lin (1982)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	3.3–333.3 µg/plate	Negative[d,n,o]	Haworth et al. (1983)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100	0.05–100 µl (53.5–107 000 µg/plate)[k]	Negative[b]	Rockwell & Raw (1979)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0.5–500 µg/plate	Negative[d,p,q]	To et al. (1982)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA1535	0.5–500 µg/plate	Positive[b,r] Negative[c]	To et al. (1982)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	5–500 µg/plate	Negative[d,e]	Yoshimura et al. (1981)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA97, TA98, TA100	1000 µg/ml	Negative[d,e]	Azizan & Blevins (1995)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102	0.25–6 µmol/l per plate (41–985 µg/plate)[a]	Negative[d,e,s]	Schiestl et al. (1989)
1529	Eugenol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	3.3–333.3 µg/plate	Negative[d,e]	National Toxicology Program (1983)
1529	Eugenol	Reverse mutation	*E. coli* WP2 uvrA (trp⁻)	60, 120, 300 or 600 µg/plate	Negative[c,h] Negative[b,e,h]	Sekizawa & Shibamoto (1982)
1529	Eugenol	DNA repair	*Bacillus subtilis* M45 (rec⁻) and H17 (rec⁺)	1–100 mg/disc (1000–100 000 µg/disc)	Negative	Yoshimura et al. (1981)

Table 7 (contd)

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
1529	Eugenol	DNA repair	*Bacillus subtilis* M45 (rec$^-$) and H17 (rec$^+$)	400 µg/disc	Positive[c]	Sekizawa & Shibamoto (1982)
1529	Eugenol	DNA repair	*Bacillus subtilis* M45 (rec$^-$) and H17 (rec$^+$)	21 µg/disc	Negative	Oda et al. (1979)
1529	Eugenol	Forward mutation	L5178Y mouse lymphoma cells	21.3 µg/ml	Positive[c,g]	Tennant et al. (1987)
1529	Eugenol	Forward mutation	L5178Y mouse lymphoma cells	20–120 nl/ml (21–128 µg/ml)[k]	Positive[c,t]	Myhr & Caspary (1991)
1529	Eugenol	Sister chromatid exchange	Peripheral human lymphocytes	0–0.5 mmol/l (0–82 µg/ml)[a]	Negative	Jansson et al. (1986)
1529	Eugenol	Sister chromatid exchange	Chinese hamster ovary cells	273–326 µg/ml	Positive[b,u]	Galloway et al. (1987)
1529	Eugenol	Sister chromatid exchange	Chinese hamster ovary cells	11–123 µg/ml	Weakly positive[c,v]	Galloway et al. (1987)
1529	Eugenol	Sister chromatid exchange	Syrian hamster embryo cells	0.00003–0.001% v/v (0.3–10 µg/ml)[k]	Weakly positive[v]	Fukuda (1987)
1529	Eugenol	Sister chromatid exchange	Chinese hamster ovary cells	75–326 µg/ml	Positive[d]	National Toxicology Program (1983)
1529	Eugenol	Sister chromatid exchange	Chinese hamster ovary cells	75 µg/ml	Positive[d,g]	Tennant et al. (1987)
1529	Eugenol	Chromosomal aberrations	Chinese hamster fibroblast cells	125 µg/ml	Negative[w] Positive[x]	Ishidate et al. (1984)
1529	Eugenol	Chromosomal aberrations	Chinese hamster ovary cells	198–300 µg/ml	Negative[c,h]	Galloway et al. (1987)
1529	Eugenol	Chromosomal aberrations	Chinese hamster ovary cells	201–324 µg/ml	Weakly positive[b,y]	Galloway et al. (1987)
1529	Eugenol	Chromosomal aberrations	Chinese hamster ovary cells	50, 100 or 200 µg/ml	Negative[z]	Stich et al. (1981)
1529	Eugenol	Chromosomal aberrations	Chinese hamster ovary cells	0.8–1.6 mmol/l (131–263 µg/ml)[a]	Positive[b,aa]	Bean et al. (1992)

Table 7 (contd)

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
1529	Eugenol	Chromosomal aberrations	Chinese hamster ovary cells	1.2, 1.4 or 1.6 mmol/l (197, 230 or 263 μg/ml)[a]	Positive[b,h,bb]	Bean & Galloway (1993)
1529	Eugenol	Chromosomal aberrations	Chinese hamster ovary cells	198–324 μg/ml	Positive[b]	National Toxicology Program (1983)
1529	Eugenol	Chromosomal aberrations	Chinese hamster ovary cells	300 μg/ml	Positive[b,g]	Tennant et al. (1987)
1529	Eugenol	Unscheduled DNA synthesis	Male Fischer 344 rat hepatocytes	0.1–1000 μmol/l (0.01642–164.2 μg/ml)[a]	Negative	Burkey et al. (2000)
1529	Eugenol	Unscheduled DNA synthesis	Female B6C3F$_1$ mouse hepatocytes	0.1–1000 μmol/l (0.01642–164.2 μg/ml)[a]	Negative	Burkey et al. (2000)
1529	Eugenol	Unscheduled DNA synthesis	Male Fischer 344 rat hepatocytes	10–6–10–3 mol/l (0.1642–164.2 μg/ml)[a]	Negative[cc]	Howes et al. (1990)
1529	Eugenol	Unscheduled DNA synthesis	Syrian hamster embryo cells	0.00003–0.0001% v/v (0.3–1 μg/ml)[k]	Positive[b]	Fukuda (1987)
1529	Eugenol	Unscheduled DNA synthesis	Male Sprague-Dawley rat hepatocytes	0.1, 0.25, or 0.5 mmol/l (16, 41 or 82 μg/ml)	Negative	Allavena et al. (1992)
1531	Eugenyl acetate	Unscheduled DNA synthesis	Male Sprague-Dawley rat hepatocytes	1, 2.5, 5, 10 or 15 μg/ml	Negative[dd]	San & Reece (2003)
In vivo						
1529	Eugenol	Micronucleus induction	Male Swiss CD-1 mice (bone marrow)	680 mg/kg bw/day for 15 days	Negative[ee]	Rompelberg et al. (1995)
1529	Eugenol	Micronucleus induction	Male B6C3F1 mice (bone marrow)	150, 300 or 600 mg/kg bw/day for 3 days	Negative[ff]	Shelby et al. (1993)
1529	Eugenol	Micronucleus induction	Humans (lymphocytes)	150 mg/day for 7 days (1.7 to 2.2 mg/kg bw/day)[gg]	Negative[hh]	Rompelberg et al. (1996b)
1529	Eugenol	Micronucleus induction	Male CF1 mice (bone marrow)	400 mg/kg bw	Positive[ff,ii]	Ellahueñe et al. (1994)

Table 7 (contd)

No.	Agent	End-point	Test object	Dose or concentration	Results	Reference
1529	Eugenol	Micronucleus induction	Male Sprague-Dawley rats (bone marrow)	1340 or 2680 mg/kg bw	Negative[ii]	Allavena et al. (1992)
1529	Eugenol	Micronucleus induction	Male CF1 mice (bone marrow)	100, 400 or 600 mg/kg bw	Positive[ff,kk,ll]	Ellahueñe et al. (1994)
1529	Eugenol	Micronucleus induction	Male Sprague-Dawley rats (liver and bone marrow)	1340 mg/kg bw	Negative[ii,mm]	Allavena et al. (1992)
1529	Eugenol	Micronucleus induction	Male Wistar rats (bone marrow)	500 or 1000 mg/kg bw per day for 10 days	Negative[hh,nn]	Rompelberg et al. (1996a)
1529	Eugenol	Micronucleus induction	Female Sprague-Dawley rats (bone marrow)	335, 670 or 1340 mg/kg bw	Negative[ii]	Maura et al. (1989)
1529	Eugenol	Micronucleus induction	Male ddY mice (bone marrow)	100, 200, 400 or 800 mg/kg bw	Negative[ff]	Hayashi et al. (1984)
1529	Eugenol	Micronucleus induction	Male Swiss Webster mice (bone marrow)	148[ff], 740[ff] or 14 794[hh] mg/kg bw	Positive	Woolverton et al. (1986)
1529	Eugenol	Unscheduled DNA synthesis	Male Sprague-Dawley rats (hepatocytes)	1340 or 2680 mg/kg bw	Negative[ii]	Allavena et al. (1992)
1529	Eugenol	Unscheduled DNA synthesis	Male Wistar rats (hepatocytes)	500 or 1000 mg/kg bw per day for 10 days	Negative[ii,oo]	Rompelberg et al. (1996a)
1529	Eugenol	DNA fragmentation	Female Sprague-Dawley rats (granuloma cells)	1, 5 or 10 mmol/kg bw (164, 821 or 1642 mg/kg bw)[a]	Negative[ii]	Maura et al. (1989)
1529	Eugenol	DNA fragmentation	Female Sprague-Dawley rats (liver and kidney)	1340 mg/kg bw	Negative[ii]	Maura et al. (1989)
1529	Eugenol	DNA fragmentation	Male Sprague-Dawley rats (hepatocytes)	1340 or 2680 mg/kg bw	Negative[ii,pp]	Allavena et al. (1992)
1529	Eugenol	Chromosomal aberration	Humans (lymphocytes)	150 mg/day for 7 days (1.7–2.2 mg/kg bw per day)[gg]	Negative[hh]	Rompelberg et al. (1996b)
1529	Eugenol	Mutation	Female Sprague-Dawley rats (granuloma cells)	1, 5 or 10 mmol/kg bw (164, 821 or 1642 mg/kg bw)[a]	Negative[ii]	Maura et al. (1989)

Table 7 (contd)

[a] Calculated from relative molecular mass for eugenol = 164.203
[b] With metabolic activation
[c] Without metabolic activation
[d] With and without metabolic activation
[e] Pre-incubation method
[f] Spot test
[g] Lowest dose that gave positive results or highest dose that gave negative results
[h] Cytotoxicity observed at highest dose
[i] Toxic to strains TA97, TA100 and TA104 at 3000 μg/plate
[j] Modified liquid suspension test system
[k] Calculated from density of eugenol = 1.07 g/ml
[l] Toxic to all strains at 1200 μg/plate
[m] Cytotoxicity observed at 5 μmol/plate (821 μg/plate)
[n] Toxic to all strains at 5000 μg/plate
[o] Cytotoxicity observed at 333.3 μg/plate in strains TA100 and TA1537
[p] Statistically significant in strain TA98 at 500 μg/plate
[q] Statistically significant in strain TA1537 at 10, 50, 150 and 500 μg/plate; at least twice the number of revertants per plate as in negative control only in first of three determinations
[r] 3′-Phosphoadenosine-5′-phosphosulfate included in metabolic activation mix
[s] No effects with or without metabolic activation in strains TA97 and TA100; weak effects in strains TA98 and TA102
[t] Cytotoxicity observed at 64 and 128 μg/ml
[u] Increases at doses that caused severe cell cycle delay
[v] Statistically significant, but induced level not twice that in the control
[w] A 24 h; dose is highest non-cytotoxic dose used
[x] At 48 h; dose is that at which maximum effect obtained
[y] In the first trial, cells fixed at about 10.5 h showed a small increase in chromosomal aberrations at the highest dose; in the second trial (with a harvesting time of 20 h), clear increase observed.
[z] Slight increase in incidence at 200 μg/ml (2.0%) relative to controls (0.8%)

Table 7 (contd)

[aa] In the first trial, with harvesting 15.0 and 24.5 h after treatment initiation, cell viability was 57% and 37% that of controls after incubation with 0.8 mmol/l of eugenol, and 53% and 43% that of controls after incubation with 1.2 mmol/l of eugenol. The percentage of aberrant cells increased to a maximum of 25.5% at harvesting 15 h after incubation with 0.8 mmol/l of eugenol. In the second trial, the aberration yield shifted with the cytotoxicity, as concentrations of 1.2–1.6 mmol/l of eugenol was as cytotoxic as 0.8 mmol/l in the first trial. Maximum percentage of aberrant cells (26.5%) on harvesting 17 h after incubation with 1.6 mmol/l of eugenol

[bb] Cells harvested 20–21 h or 42–44 h after beginning of 3-h treatment with eugenol. Severe cell cycle delay observed 20 h after incubation with 1.6 mmol/l of eugenol, and chromosomal aberrations accompanied by a reduction in cell counts to about 50% that of controls

[cc] Cytotoxicity observed at 500 µmol/l

[dd] Cytotoxicity observed at 10 and 15 µg/ml

[ee] Administered in diet

[ff] Administered by intraperitoneal injection

[gg] Range of doses calculated on basis of body weight range of volunteers: 68–88 kg

[hh] Administered orally

[ii] Sampling 24, 30 or 48 h after intraperitoneal injection

[jj] Administered by gavage

[kk] Sampled once, 30 h after intraperitoneal injection

[ll] Significant increases at 400 and 600 mg/kg bw of eugenol

[mm] Rats underwent two-thirds hepatectomy 20 h before eugenol administered

[nn] Slight, not statistically significant increase in percentage of polychromatic erythrocytes at 1000 mg/kg bw per day

[oo] Hepatocytes from rats pre-treated with 500 mg/kg bw of eugenol and then exposed in vitro to dimethyl sulfoxide had significantly lower mean net nuclear grain count than control hepatocytes (dimethyl sulfoxide); no results for 1000 mg/kg bw eugenol plus dimethyl sulfoxide

[pp] Observed 4 and 20 h after seeding

Shibamoto (1982) reported positive results, observed as evidence of preferential lethality of eugenol for M45 rec$^-$ cells. The authors noted, however, that as the eugenol sample used was somewhat oily it did not diffuse effectively in the aqueous agar layer. Therefore, the H17 rec$^+$ cells (which had a doubling time of 48 min, to be compared with 75 min for M45 rec$^-$) might have grown before the sample diffused effectively, which would have resulted in smaller inhibition zones than in the M45 rec$^-$ cells.

In an assay for forward mutation in mouse lymphoma cells, eugenol was mutagenic in L5178Y cells at concentrations of 21.3–128 μg/ml (0.13–0.78 mmol/l) (Tennant et al., 1987; Myhr & Caspary, 1991). The concentrations used, however, were highly cytotoxic. In the two trials conducted by Myhr and Caspary (1991), a negative correlation was found between mutation frequency and relative total growth. In the first trial, the relative total growth was 16–28% at a eugenol concentration of 80 nl/ml and 5–11% at a concentration of 120 nl/ml. In the second trial, the relative total growth was 5–30% at a concentration of 40 nl/ml and 4% at 60 nl/ml. The mutation frequency increased by 4.2- and 2.2-fold at the highest doses tested in the first and second trials, respectively. According to Myrh and Caspary (1991), the interaction between eugenol and the cells was not well controlled, and additional studies are necessary to characterize the dose–response relation better.

Assays for sister chromatid exchange in mammalian cells conducted with eugenol gave equivocal results (National Toxicology Program, 1983; Jansson et al., 1986; Fukuda, 1987; Galloway et al., 1987; Tennant et al., 1987). In human peripheral lymphocytes, no sister chromatid exchange was induced at eugenol concentrations $\leq$ 82.1 μg/ml (0.5 mmol/l) (Jansson et al., 1986). When 0.3–10 μg/ml [0.00003–0.001% (v/v)] of eugenol were incubated with Syrian hamster embryo cells, a significant increase ($p < 0.001$) in sister chromatid exchange was reported but at less than twice the level found in negative controls (Fukuda, 1987). Weak induction of sister chromatid exchange was reported in Chinese hamster ovary cells incubated with 11–123 μg/ml (0.07–0.75 mmol/l) of eugenol in the absence of metabolic activation, and positive results were observed after incubation with 273–326 μg/ml (1.7–2.0 mmol/l) of eugenol in the presence of metabolic activation. The authors commented that the increase in sister chromatid exchange observed with and without metabolic activation occurred at doses that caused severe cell cycle delay (Galloway et al., 1987). Sister chromatid exchange was induced in Chinese hamster ovary cells incubated with 75–326 μg/ml (0.5–2.0 mmol/l) of eugenol in the absence and presence of metabolic activation (National Toxicology Program, 1983; Tennant et al., 1987).

Equivocal results were reported in standard assays for chromosomal aberration (Stich et al., 1981; National Toxicology Program, 1983; Ishidate et al., 1984; Galloway et al., 1987; Tennant et al., 1987; Bean et al., 1992; Bean & Galloway, 1993). In a study designed to investigate the optimal sampling time for detecting chromosomal aberrations in Chinese hamster ovary cells, significant increases in chromosomal aberrations were reported at eugenol concentrations of 131–263 μg/ml (0.8–1.6 mmol/l) in the presence of metabolic activation (Bean et al., 1992). In the first of two trials, when cells were harvested 15 and 24.5 h after the beginning of treatment, cell viability was 57% and 37% that of controls, respectively, after incubation with 0.8 mmol/l of eugenol, and 53% and 43% that of controls after incubation with 1.2 mmol/l of eugenol. The percentage of aberrant cells increased to a maximum of 25.5% on harvesting 15 h after incubation with 0.8 mmol/l of eugenol.

In the second trial, the aberration yield shifted with the cytotoxicity, as concentrations of 1.2–1.6 mmol/l of eugenol were as cytotoxic as 0.8 mmol/l in the first trial. The maximal increase in the percentage of aberrant cells (26.5%) was found at harvest of cells 17 h after incubation with 1.6 mmol/l of eugenol.

In a subsequent study with a similar purpose, Chinese hamster ovary cells incubated with 197, 230 or 263 μg/ml (1.2, 1.4 or 1.6 mmol/l) of eugenol for 3 h and harvested 20 or 44 h after the beginning of treatment showed significant increases in chromosomal aberrations. Severe cell cycle delay was observed 20 h after incubation with 263 μg/ml (1.6 mmol/l) of eugenol, and the chromosomal aberrations were accompanied by a reduction in cell count to about 50% that of controls (Bean & Galloway, 1993).

Chromosomal aberrations occurred when Chinese hamster fibroblast cells were incubated for 48 h with ≤ 125 μg/ml (0.77 mmol/l) of eugenol; however, negative results were reported after only 24 h of incubation. The dose at which structural aberrations were detected in 20% of metaphases was 14.8 mg/ml (Ishidate et al., 1984). In another study, no chromosomal aberrations were found in Chinese hamster ovary cells incubated with 50 or 100 μg/ml (0.31 or 0.61 mmol/l) of eugenol; however, a slight increase (2.0%) in the incidence of chromosomal aberrations was obtained at the highest dose, 200 μg/ml (1.22 mmol/l) when compared with controls (0.8%) (Stich et al., 1981). Negative results were reported with eugenol in a standard assay for chromosomal aberrations without S9 activation at concentrations ≤ 300 μg/ml (1.8 mmol/l), which was a toxic concentration. in the presence of S9 activation, weakly positive results were found at concentrations of 201–324 μg/ml (1.2–2.0 mmol/l) (Galloway et al., 1987). Induction of chromosomal aberrations was reported in Chinese hamster ovary cells exposed to eugenol at concentrations of 300–324 μg/ml (1.8–2.0 mmol/l) in the presence of metabolic activation (National Toxicology Program, 1983; Tennant et al., 1987). Hepatocytes isolated from male Fischer 344 rats and female B6C3F$_1$ mice showed no unscheduled DNA synthesis after incubation with 0.01642, 0.1642, 1.642, 16.42 or 164.2 μg/ml (0.1, 1, 10, 100 or 1000 μmol/l) of eugenol. The LC_{50} values for eugenol in this study were 49.3 μg/ml (300 μmol/l) for rat hepatocytes and 32.8 μg/ml (200 μmol/l) for mouse hepatocytes; no unscheduled DNA synthesis induction was observed at these doses (Burkey et al., 2000). In a similar study, no unscheduled DNA synthesis was reported in rat hepatocytes incubated in the presence of 0.1642–164.2 μg/ml (10–6–10–3 mol/l) of eugenol. Cytotoxicity occurred, as indicated by increased activity of lactate dehydrogenase, at 82.1 μg/ml (5×10^{-4} mol/l) (Howes et al., 1990). No unscheduled DNA synthesis was reported when hepatocytes from male Sprague-Dawley rats were exposed for 20 h to 16, 41 or 82 μg/ml (0.1, 0.25 or 0.5 mmol/l) of eugenol (Allavena et al., 1992). Fukuda (1987), however, reported positive results with 0.3–1 μg/ml [0.00003–0.0001% (v/v)] in an assay for unscheduled DNA synthesis in Syrian hamster embryo cells in the presence of metabolic activation.

When 1, 2.5, 5, 10 or 15 μg/ml of eugenyl acetate were incubated with isolated rat hepatocytes, no unscheduled DNA synthesis was reported. The compound was cytotoxic at a concentration of 33 μg/ml, as evidenced by lactate dehydrogenase leakage (San & Reece, 2003).

In vivo

In a standard assay for micronucleus induction in vivo, groups of eight male CF1 mice were given 100, 400 or 600 mg/kg bw of eugenol by intraperitoneal injection.

The lowest dose did not induce micronucleated polychromatic erythrocytes in the bone marrow of the mice, but the intermediate and high doses had significantly increased the frequency ($p < 0.01$) by 30 h after treatment. In groups of eight male CF1 mice given 400 mg/kg bw of eugenol by intraperitoneal injection and killed 24, 30 or 48 h later, significant increases ($p < 0.01$) in the frequency of micronucleated polychromatic erythrocytes were reported, regardless of the time of sacrifice (Ellahueñe et al., 1994).

Groups of 12 adult male Swiss-Webster mice received 148 or 740 mg/kg bw of eugenol by intraperitoneal injection or 14 794 mg/kg bw by oral intubation. Bone marrow isolated from the femur of each animal showed a significant increase in micronucleated polychromatic erythrocytes over that in saline controls; however, intraperitoneal administration induced a higher frequency (18.5–20.5%) than oral administration (5.7%), with a considerably lower concentration of test material (Woolverton et al., 1986).

Studies in various strains of mice (Hayashi et al., 1984; Shelby et al., 1993; Rompelberg et al., 1995) and rats (Maura et al., 1989; Allavena et al., 1992; Rompelberg et al., 1996a) and in human lymphocytes (Rompelberg et al., 1996b) showed no evidence of mutagenicity. In male ddY mice given eugenol at 100, 200, 400 or 800 mg/kg bw by intraperitoneal injection, no increase in the frequency of micronucleated polychromatic erythrocytes was reported (Hayashi et al., 1984). Male CD-1 mice fed a diet containing 0.4% eugenol daily (average intake, about 680 mg/kg bw per day) for 15 days showed no increase in the incidence of micronuclei in bone marrow (Rompelberg et al., 1995). No micronuclei were induced when male B6C3F1 mice received 150, 300 or 600 mg/kg bw per day by intraperitoneal injection for 3 consecutive days (Shelby et al., 1993).

Male albino Sprague-Dawley rats showed no induction of micronuclei in bone marrow after administration of 1340 or 2680 mg/kg bw of eugenol by gavage by three protocols. The first involved administering 1340 mg/kg bw of eugenol 20 h after a partial hepatectomy, followed by sacrifice at 48 h, while the second protocol involved administering 2680 mg/kg bw of eugenol 30 and 6 h before sacrifice. In the third protocol, 1340 mg/kg bw of eugenol were administered, followed by sacrifice at 2 h. No positive dose-dependent relation was observed with these protocols. Administration of 1340 or 2680 mg/kg bw by gavage did not induce DNA fragmentation in primary cultures of rat hepatocytes 4 and 20 h after seeding (Allavena et al., 1992).

Groups of four female Sprague-Dawley rats received 335, 670 or 1340 mg/kg bw of eugenol orally in an aqueous suspension, half of the dose being administered 30 h before sacrifice and the second half 6 h before sacrifice. No micronucleated polychromatic erythrocytes were induced in bone marrow. At 1340 mg/kg bw, eugenol did not induce DNA fragmentation in the liver or kidney after 2, 24 or 48 h of treatment. Furthermore, administration of 1.5, 5 or 10 mmol/kg bw (164.2, 821.0 and 1642 mg/kg bw, respectively) of eugenol by gavage did not significantly increase the mutation frequency or induce DNA fragmentation in granuloma cells. The authors proposed that the mutagenic and clastogenic activity of eugenol observed in mammalian cells in vitro might be due to the absence of the necessary detoxifying enzymes that exist in vivo (Maura et al., 1989).

In a similar study, male Wistar rats were given 0, 500 or 1000 mg/kg bw per day of eugenol in corn oil suspension by gavage for 10 days. No significant increase in the occurrence of bone-marrow micronucleated polychromatic erythrocytes was

reported at either dose when compared with controls. Under similar conditions, primary hepatocyte cultures obtained from male Wistar rats given 500 mg/kg bw per day of eugenol orally for 10 days showed no unscheduled DNA synthesis. The authors did not report results for unscheduled DNA synthesis in hepatocytes of rats given 1000 mg/kg bw eugenol (Rompelberg et al., 1996a).

In a 7-day, placebo-controlled, cross-over study with a 1-week washout period, 10 healthy male volunteers received a placebo or 150 mg of eugenol daily in the form of a capsule (50 mg/capsule) taken orally 3 times a day. On the basis of the body weights of the volunteers (68–88 kg), this dose provided about 1.7–2.2 mg/kg bw per day of eugenol. Aliquots of blood were collected on days 8 and 22 of the study and analysed for micronucleus induction and chromosomal aberrations. Eugenol did not increase the background level of micronuclei or chromosomal aberrations in human lymphocytes (Rompelberg et al., 1996b).

Conclusion

One representative agent of this group, eugenol (No. 1529), gave consistently negative results in assays for reverse mutation in various strains of *S. typhimurium* and *E. coli*. Generally, negative results were also found for DNA repair in *B. subtilis* M45 (rec^-) and H17 (rec^+) cells; the one positive finding for DNA repair occurred at a concentration of 400 μg/disc, while a similar study with higher concentrations (≤ 100 000 μg/disc) yielded negative results. In assays for unscheduled DNA synthesis in rat and mouse hepatocytes in vitro, no genotoxic activity was observed at concentrations ≤ 164.2 μg/ml of eugenol and ≤ 15 μg/ml of eugenyl acetate; however, one positive result was reported with eugenol in Syrian hamster embryo cells at concentrations ≤ 1 μg/ml. Assays in mammalian cells in vitro in which mutagenicity was found (forward mutation in mouse lymphoma cells, sister chromatid exchange and chromosomal aberrations) were performed at concentrations that resulted in cytotoxicity or severe cell cycle delay. Mammalian cells in culture might not have the metabolic pathways of detoxication available to counter such toxicity. Assays for mutagenicity (micronuclei, chromosomal aberrations and mutation) and genotoxicity (unscheduled DNA synthesis and DNA fragmentation) in vivo generally gave negative results, even at very high doses of eugenol (≤ 800 mg/kg bw by intraperitoneal injection, ≤ 2680 mg/kg bw orally). The two reports of micronucleus induction involved doses as high as 740 mg/kg bw given by intraperitoneal injection and 14 794 mg/kg bw given orally. The available results indicate that eugenol and other hydroxypropylbenzene derivatives are unlikely to pose a significant mutagenic or genotoxic risk to humans under the intended conditions of their use as flavouring agents.

3. REFERENCES

Allavena, A., Martelli, A., Robbiano, L. & Brambilla G. (1992) Evaluation in a battery of in vivo assays of four in vitro genotoxins proved to be noncarcinogens in rodents. *Teratog. Carcinog. Mutag.*, **12**, 31–41.

Amonkar, A.J., Nagabhushan, M., D'Souza, A.V. & Bhide, S.V. (1986) Hydroxychavicol: a new phenolic antimutagen from betel leaf. *Food Chem. Toxicol.*, **24**, 1321–1324.

Azizan, A. & Blevins, R.D. (1995) Mutagenicity and antimutagenicity testing of six chemicals associated with the pungent properties of specific spices as revealed by the Ames Salmonella microsomal assay. *Arch. Environ. Contam. Toxicol.*, **28**, 248–258.

Bär, V.F. & Griepentrog, F. (1967) [Where we stand concerning the evaluation of flavoring substances from the viewpoint of health]. *Medizin. Ernahr.*, **8**, 244–251 (in German).

Bean, C.L. & Galloway, S.M. (1993) Evaluation of the need for a late harvest time in the assay for chromosome aberrations in Chinese hamster ovary cells. *Mutat. Res.*, **292**, 3–16.

Bean, C.L., Armstrong, M.J. & Galloway, S.M. (1992) Effect of sampling time on chromosome aberration yield for 7 chemicals in Chinese hamster ovary cells. *Mutat. Res.*, **65**, 31–44.

Bennett, A., Stamford, I.F., Tavares, I.A., Jacobs, S., Capasso, F., Mascolo, N., Autore, G., Romano, V. & DiCarlo, G. (1988) The biological activity of eugenol, a major constituent of nutmeg (*Myristica fragrans*): studies on prostaglandins, the intestines and other tissues. *Phytother. Res.*, **2**, 124–130.

Beroza, M., Inscoe, M.N., Schwartz, P.H. Jr, Keplinger, M.L. & Mastri, C.W. (1975) Acute toxicity studies with insect attractants. *Toxicol. Appl. Pharmacol.*, **31**, 421–429.

Bolton, J.L., Comeau, E. & Vukomanovic, V. (1995) The influence of 4-alkyl substituents on the formation and reactivity of 2-methoxy-quinone methides: evidence that extended π-conjugation dramatically stabilizes the quinone methide formed from eugenol. *Chem.-Biol. Interactions*, **95**, 279–290.

Boutin, J.A., Siest, G., Batt, A.M., Solheim, E. & Scheline, R.R. (1983) Studies of UDP-glucuronosyltransferase activity toward eugenol, using gas chromatographic method of measurment. *Analyt. Biochem.*, **135**, 201–207.

Boutin, J.A., Thomassin, J., Siest, G. & Cartier, A. (1985) Heterogeneity of hepatic microsomal UDP glucuronosyltransferase activities. *Biochem. Pharmacol.*, **34**, 2235–2249.

Burkey, J.L., Sauer, J.-M., McQueen, C.A. & Sipes, I.G. (2000) Cytotoxicity and genotoxicity of methyleugenol and related congeners—a mechanism of activation for methyleugenol. *Mutat. Res.*, **453**, 25–33.

Castro, D.J., Sweet, C.J., Kuester, R.K. & Sipes, I.G. (2004) Hydrolysis of isoeugenyl-acetate and eugenyl-acetate by rat and human hepatic microsomes. *Toxicologist*, **78**, 1446.

Cramer, G.M., Ford, R.A. & Hall, R.L. (1978) Estimation of toxic hazard—a decision tree approach. *Food Cosmet. Toxicol.*, **16**, 255–276.

Delaforge, M., Janiaud, P., Maume, B.F. & Padieu, P. (1978) Direct evidence of epoxide metabolic pathway for natural allylbenzene compounds in adult rat liver cell culture. *Recent Dev. Mass Spectrom. Biochem. Med.*, **1**, 521–539.

Delaforge, M., Janiaud, P., Levi, P. & Morizot, J.P. (1980) Biotransformation of allylbenzene analogues in vivo and in vitro through the epoxide-diol pathway. *Xenobiotica*, **10**, 737–744.

Dorange, J.-L., Delaforge, M., Janiaud, P. & Padieu, P. (1977) [Mutagenicity of the metabolites of the epoxide diol pathway of safrole and analogs. Study on *Salmonella typhimurium*.] *Soc. Biol. Dijon*, **171**, 1041–1048 (in French, with English summary).

Douglas, G.R., Nestmann, E.R., Betts, J.L., Mueller, J.C., Lee, E.G.-H., Stich, H.F., San, R.H.C., Brouzes, R.J.P., Chmelauskas, A., Paavila, H.D. & Walden, C.C. (1980) Mutagenic activity in pulp mill effluents. In: *Water Chlorination, Environmental Impact and Health Effects*, Vol. 3, Ch. 76, pp. 865–880. Editors, publishers?

Eder, E., Neudecker, T., Lutz, D. & Henschler, D. (1980) Mutagenic potential of allyl and allylic compounds. Structure–activity relationship as determined by alkylating and direct in vitro mutagenic properties. *Biochem. Pharmacol.*, **29**, 993–998.

Eder, E., Neudecker, T., Lutz, D. & Henschler, D. (1982) Correlation of alkylating and mutagenic activities of allyl and allylic compounds: standard alkylation test vs. kinetic investigation. *Chem.-Biol. Interactions*, **38**, 303–315.

Ellehueñe, M.F., Perez-Alzola, L.P., Orellana-Vandebenito, M., Muñoz, C. & Lafuente-Valdebenito, N. (1994) Genotoxic evaluation of eugenol using the bone marrow micronucleus assay. *Mutat. Res.*, **320**, 175–180.

Fischer, I.U., von Unruh, G.E. & Dengle, H.J. (1990) The metabolism of eugenol in man. *Xenobiotica*, **20**, 209–222.

Fishbeck, W.A., Langer, R.R. & Kociba, R.J. (1975) Elevated urinary phenol levels not related to benzene exposure. *Am. Ind. Hyg. Assoc. J.*, **36**, 820–824

Florin, I., Rutberg, L., Curvall, M. & Enzell, C.R. (1980) Screening of tobacco smoke constituents for mutagenicity using the Ames' test. *Toxicology*, **18**, 219–232.

Food & Drug Administration (1993) *Priority-based Assessment of Food Additives (PAFA) Database*, Washinton DC, Center for Food Safety and Applied Nutrition, p. 58.

Fukuda, S. (1987) [Assessment of the carcinogenic hazard of 6 substances used in dental practices.] *Shikagu*, **74**, 1365–1384 (in Japanese with English sumary).

Galloway, S.M., Armstrong, M.J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A.D., Nakamura, F., Ahmed, M., Duk, S., Rimpo, J., Margolin, B.H., Resnick, M.A., Anderson, B. & Zeiger, E. (1987) Chromosome aberration and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. *Environ. Mol. Mutag.*, **10**, 1–175.

Graffner-Nordberg, M., Karin, S., Anders, T. & Anders, H. (1998) Synthesis and enzymes hydrolysis of esters, constituting simple models of soft drugs. *Chem. Pharm. Bull.*, **46**, 591–601.

Green, M.D. & Tephly, T.R. (1996) Glucuronidation of amines and hydroxylated xenobiotics and endobiotics catalyzed by expressed human UGT1.4 protein. *Drug Metab. Disposition*, **24**, 356–363.

Green, N.R. & Savage, J.R. (1978) Screening of safrole, eugenol, their ninhydrin positive metabolites and selected secondary amines for potential mutagenicity. *Mutat. Res.*, **57**, 115–121.

Gruebner, I., Klinger, W. & Ankermann, H. (1972) Various substances and substance classes with inducer properties. *Arch. Int. Pharmacodyn.*, **196**, 1–9.

Grundschober, F. (1977) Toxicological assessment of flavouring esters. *Toxicology*, **8**, 387–390.

Guenthner, T.M. & Luo, G. (2001) Investigation of the role of the 2′,3′-epoxidation pathway in the bioactivation and genotoxicity of dietary allylbenzene analogs. *Toxicology*, **160**, 47–58.

Hagan, E.C., Jenner, P.M., Jones, W.I., Fitzhugh, O.G., Long, E.L., Brouwer, J.G. & Webb, W.K. (1965) Toxic properties of compounds related to safrole. *Toxicol. Appl. Pharmacol.*, **7**, 18–24

Hagan, E.C., Hansen, W.H., Fitzhugh, O.G., Jenner, P.M., Jones, W.I., Taylor, J.M., Long, E.L., Nelson, A.A. & Brouwer, J.B. (1967) Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Food Cosmet. Toxicol.*, **5**, 141–157.

Haseman, J.K., Huff, J. & Boorman, G.A. (1984) Use of historical control data in carcinogenicity studies in rodents. *Toxicol. Pathol.*, **12**, 126–135.

Haseman, J.K., Hailey, J.R. & Morris, R.W. (1998) Spontaneous neoplasm incidences in Fischer 344 rats and B6C3F1 mice in two-year carcinogenicity studies: a National Toxicology Program update. *Toxicol. Pathol.*, **26**, 428–441.

Haworth, S., Lawlor, T., Mortelmans, K., Speck, W. & Zeiger, E. (1983) *Salmonella* mutagenicity test results for 250 chemicals. *Environ. Mutag.*, **5** (Suppl. 1), 3–142.

Hayashi, M., Sofuni, T. & Ishidate, M., Jr (1984) A pilot experiment for the micronucleus test. The multi-sampling at multi-dose levels method. *Mutat. Res.*, **141**, 165–169.

Heymann, E. (1980) Carboxylesterases and amidases. In: Jakoby, W.B., ed., *Enzymatic Basics of Detoxication*, 2nd Ed., New York, Academic Press, pp. 291–323.

Hirose, M., Masuda, A., Imaida, K., Kagawa, M., Tsuda, H. & Ito, N. (1987) Induction of forestomach lesions in rats by oral administrations of naturally occurring antioxidants for 4 weeks. *Jpn. J. Cancer Res.*, **78**, 317–321.

Hosokawa, M., Watanabe, N., Tsukada, E., Fukumoto, M., Chiba, K., Takeya, M., Imai, T., Sasaki, Y.F. & Sato, T. (2001) Multiplicity of carboxylesterase isozymes in mammals and humans: role in metabolic activation of prodrugs. *Yakubutsu Dotai* (Xenobiotic Metab. Disposition), **16** (Suppl.), 92–93.

Howes, A.J., Chan, V.S.W. & Caldwell, J. (1990) Structure-specificity of the genotoxicity of some naturally occurring alkenylbenzenes determined by the unscheduled DNA synthesis assay in rat hepatocytes. *Food Chem. Toxicol.*, **28**, 537–542.

International Organization of the Flavor Industry (1995) European inquiry on volume use. Private communication to the Flavor and Extract Manufacturers Association of the United States. Submitted to WHO by the Flavor and Extract Manufacturers Association of the United States, Washington DC, USA.

Ishidate, M., Jr, Sofuni, T., Yoshikawa, K., Hayashi, M., Nohmi, T., Sawada, M. & Matsuoka, A. (1984) Primary mutagenicity screening of food additives currently used in Japan. *Food Chem. Toxicol.*, **22**, 623–636.

Jansson, T., Curvall, M., Hedin, A. & Enzell, C.R. (1986) In vitro studies of biological effects of cigarette smoke condensate. II. Induction of sister-chromatid exchanges in human lymphocytes by weakly acidic, semivolatile constituents. *Mutat. Res.*, **169**, 129–139.

Jenner, P.M., Hagan, E.C., Taylor, J.M., Cook, E.L. & Fitzhugh, O.G. (1964) Food flavorings and compounds of related structure. I. Acute oral toxicity. *Food Cosmet. Toxicol.*, **2**, 327–343.

Junge, W. & Heymann, E. (1979) Characterization of the isoenzymes of pig liver carboxylesterase. II. Kinetic studies. *Eur. J. Biochem.*, **95**, 519–525.

Leclerc, S., Heydel, J.-M., Amossé, V., Gradinaru, D., Cattarelli, M., Artur, Y., Goudonnet, H., Magdalou, J., Netter, P., Pelczar, H. & Minn, A. (2002) Glucuronidation of odorant molecules in the rat olfactory system. Activity, expression and age-linked modifications of UDP-glucuronosyltransferase isoforms, UGT1A6 and UGT2A1 and relation to mitral cell activity. *Mol. Brain Res.*, **107**, 201–213.

Lucas, C.D., Putnam, J.M. & Hallagan, J.B. (1999) *1995 Poundage and Technical Effects Update Survey*, Flavor and Extract Manufacturers Association of the United States, Washington DC, USA.

Luo, G., Qato, M.K. & Guenthner, T.M. (1992) Hydrolysis of the 2′,3′-allylic epoxides of allylbenzene, estragole, eugenol, and safrole by both microsomal and cytosolic epoxide hydrolases. *Drug Metab. Disposition*, **20**, 440–445.

Maronpot, R.R., Haseman, J.K., Boorman, G.A., Eustis, S.E., Rao, G.N. & Huff, J.E. (1987) Liver lesions in B6C3F1 mice: the National Toxicology Program experience and position. *Arch. Toxicol.*, **Suppl. 10**, 10–26.

Maura, A., Pino, A. & Ricci, R. (1989) Negative evidence in vivo of DNA-damaging, mutagenic and chromosomal effects of eugenol. *Mutat. Res.*, **227**, 125–129.

Miller, E.C., Swanson, A.B., Phillips, D., Fletcher, L.T., Liem, A. & Miller, J.A. (1983) Structure–activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Res.*, **43**, 1124–1134.

Miller, E.G., Washington, V.H., Bowles, W.H. & Zimmermann, E.R. (1986) Mutagenic potential of some chemical components of dental materials. *Dental Mater.*, **2**, 163–165.

Mizutani, T., Satoh, K., Nomura, H. & Nakanishi, K. (1991) Hepatotoxicity of eugenol in mice depleted of glutathione by treatment with DL-buthione sulfoximine. *Res. Commun. Chem. Pathol. Pharmacol.*, **71**, 219–230.

Moreno, O.M. (1972) Acute oral toxicity in rats (eugenol acetate). Unpublished report to the Research Institute of Fragrance Materials, Englewood Cliffs, New Jersey, USA. Submitted to WHO by the Flavor and Extract Manufacturers Association of the United States, Washington DC, USA.

Moreno, O.M. (1977) Acute oral toxicity in rats (eugenol formate). Unpublished report to the Research Institute of Fragrance Materials, Englewood Cliffs, New Jersey, USA. Submitted to WHO by the Flavor and Extract Manufacturers Association of the United States, Washington DC, USA.

Myhr, B.C. & Caspary, W.J. (1991) Chemical mutagenesis at the thymidine kinase locus in L5178Y mouse lymphoma cells: Results for 31 coded compounds in the National Toxicology Program. *Environ. Mol. Mutag.*, **18**, 51–83.

National Academy of Sciences (1982) *Poundage and Technical Effects Update of Substances Added to Food. Committee on Food Additives Survey Data*, Washington DC, Food and Nutrition Board, Institute of Medicine.

National Academy of Sciences (1987) *Poundage and Technical Effects Update of Substances Added to Food. Committee on Food Additives Survey Data*, Washington DC, Food and Nutrition Board, Institute of Medicine.

National Toxicology Program (1983) *Carcinogenesis Studies of Eugenol (CAS No. 97-53-0) in F344/N Rats and B6C3F1 Mice (Feed Studies)*,TR 223, NTP publication No. 84-1779, Research Triangle Park, North Carolina, USA, National Toxicology Program.

Nestmann, E.R., Lee, E.G.-H., Matula, T.I., Douglas, G.R. & Mueller, J.C. (1980) Mutagenicity of constituents identified in pulp and paper mill effluents using the *Salmonella*/mammalian-microsome assay. *Mutat. Res.*, **79**, 203–212.

Nijssen, B., van Ingen-Visscher, K. & Donders, J. (2004) *Volatile Compounds in Food 8.1*, Zeist, Netherlands, Centraal Instituut Voor Voedingsonderzioek TNO. http://www.voeding.tno.nl/vcf/VcfNavigate.cfm.

Oda, Y., Hamono, Y., Inoue, K., Yamamoto, H., Niihara, T. & Kunita, N. (1979) [Mutagenicity of food flavors in bacteria.] *Shokuhin Eisei Hen*, **9**, 177–181 (in Japanese with English summary).

Orstavik, D. & Hongslo, J.K. (1985) Mutagenicity of endodontic sealers. *Biomaterials*, **6**, 129–132.

Pool, B.L. & Lin, P.Z. (1982) Mutagenicity testing in the *Salmonella* typhimurium assay of phenolic compounds and phenolic fractions obtained from smokehouse smoke condensates. *Food Chem. Toxicol.*, **20**, 383–391.

Rapson, W.H., Nazar, M.A. & Butsky, V.V. (1980) Mutagenicity produced by aqueous chlorination of organic compounds. *Bull. Environ. Contam. Toxicol.*, **24**, 590–596.

Rockwell, P. & Raw, I. (1979) A mutagenic screening of various herbs, spices, and food additives. *Nutr. Cancer*, **1**, 10–15.

Rompelberg, C.J.M., Verhagen, H. & van Bladeren, P.J. (1993) Effects of the naturally occurring alkylbenzenes eugenol and trans-anethole on drug-metabolizing enzymes in the rat liver. *Food Chem. Toxicol.*, **31**, 637–645.

Rompelberg, C.J.M., Stenhuis, W.H., de Vogel, N., van Osenbruggen, V., Schouten, A. & Verhagen H. (1995) Antimutagenicity of eugenol in the rodent bone marrow micronucleus test. *Mutat. Res.*, **346**, 69–75.

Rompelberg, C.J.M., Evertz, S.J.C.J., Bruijntjes-Rozier, G.C.D.M., van den Heuvel, P.D. & Verhagen, H. (1996a) Effect of eugenol on the genotoxicity of established mutagens in the liver. *Food Chem. Toxicol.*, **34**, 33–42.

Rompelberg, C.J.M., Vogels, J.T.W.E., de Vogel N., Bruijntjes-Rozier, G.C.D.M., Stenhuis, W.H., Bogaards, J.J.P. & Verhagen, H. (1996b) Effect of short-term dietary administration of eugenol in humans. *Hum. Exp. Toxicol.*, **15**, 129–135.

San, R.H.C. & Reece, J.D. (2003) Unscheduled DNA synthesis in mammalian cells in vitro. Study No. AA51FW-FZ, AA52CV.380.BTL. BioReliance, Rockville, Maryland, USA. Unpublished report to the Research Institute for Fragrance Materials, Woodcliff Lake, New Jersey, USA. Submitted to WHO by the Flavor and Extract Manufacturers Association of the United States, Washington DC, USA.

Schiestl, R.H., Chan, W.S., Gietz, R.D., Mehta, R.D. & Hast, P.J. (1989) Safrole, eugenol and methyleugenol induce intrachromosomal recombination in yeast. *Mutat. Res.*, **224**, 427–436.

Schröder, V. & Vollmer, H. (1932) The excretion of thymol, carvacrol, eugenol and guaiacol and the distribution of these substances in the organism. *Arch. Exp. Pathol. Pharmakol.*, **168**, 331–353.

Sekizawa, J. & Shibamoto, T. (1982) Genotoxicity of safrole-related chemicals in microbial test systems. *Mutat. Res.*, **101**, 127–140.

Senafi, S.B., Clarke, D.J. & Burchell, B. (1994) Investigation of the substrate specificity of a cloned expressed human billirubin UDP-glucuronosyltransferase: UDP-sugar specificity and involvement in steroid and xenobiotic glucuronidation. *Biochem. J.*, **383**, 233–240.

Shelby, M.D., Erexson, G.L., Hook, G.L. & Tice, R.R. (1993) Evaluation of a three-exposure mouse bone marrow micronucleus protocol: results with 49 chemicals. *Environ. Mol. Mutag.*, **21**, 160–179

Sober, H.A., Hollander, F. & Sober, E.K. (1950) Toxicity of eugenol: determination of LD_{50} on rats. *Proc. Soc. Exp. Biol. Med.*, **73**, 148–151.

Stich, H.F., Stich, W. & Lam, P.P.S. (1981) Potentiation of genotoxicity by concurrent application of compounds found in betel quid: arecoline, eugenol, quercetin, chlorogenic acid and Mn^{2+}. *Mutat. Res.*, **90**, 355–363.

Stofberg, J. & Grundschober, F. (1987) Consumption ratio and food predominance of flavoring materials. *Perfum. Flavorist*, **12**, 27.

Stofberg, J. & Kirschman, J.C. (1985) The consumption ratio of flavoring materials: a mechanism for setting priorities for safety evaluation. *Food Chem. Toxicol.*, **23**, 857–860.

Sukumaran, K. & Kuttan, R. (1995) Inhibition of tobacco-induced mutagenesis by eugenol and plant extracts. *Mutat. Res.*, **343**, 25–30.

Sutton, J.D. (1986) *Metabolic Studies of Eugenol in Relation to its Safety Evaluation*, Thesis submitted for the degree of Doctor of Philosophy in the University of London.

Sutton, J.D., Sangster, S. & Caldwell, J. (1985) Dose-dependent variation in the disposition of eugenol in the rat. *Biochem. Pharmacol.*, **34**, 465–466.

Suzuki, Y., Sugiyama, K. & Furuta, H. (1985) Eugenol-mediated superoxide generation and cytotoxicity in guinea pig neutrophils. *Jpn. J. Pharmacol.*, **39**, 381–386.

Swanson, A.B., Chambliss, D.D., Blomquist, J.C., Miller, E.C. & Miller, J.A. (1979) The mutagenicities of safrole, estragole, eugenol, *trans*-anethole and some of their known or possible metabolites for *Salmonella typhimurium* mutants. *Mutat. Res.*, **60**, 143–153.

Tennant, R.W., Margolin, B.H., Shelby, M.D., Zeiger, E., Haseman, J.K., Spalding, J., Caspary, W., Resnick, M., Stasiewicz, S., Anderson, B. & Minor, R. (1987) Prediction of chemical carinogenicity in rodents from in vitro genetic toxicity assays. *Science*, **236**, 933–941.

Thompson, D., Constantin-Teodosiu, D., Norbeck, K., Svensson, B. & Moldeus, P. (1989) Metabolic activation of eugenol by myeloperoxidase and polymorphonuclear leukocytes. *Chem. Res. Toxicol.*, **2**, 186–192.

Thompson, D., Constantin-Teodosiu, D., Egestad, B., Mickos, H. & Moldeus, P. (1990) Formation of glutathione conjugates during oxidation of eugenol by microsomal fractions of rat liver and lung. *Biochem. Pharmacol.*, **39**, 1587–1595.

Thompson, D., Constantin-Teodosiu, D. & Moldeus, P. (1991) Metabolism and cytotoxicity of eugenol in isolated rat hepatocytes. *Chem.-Biol. Interactions*, **77**, 137–147.

Thompson, D.C., Barhoumi, R. & Burghardt, R.C. (1998) Comparative toxicity of eugenol and its quinone methide metabolite in cultured liver cells using kinetic fluorescence bioassays. *Toxicol. Appl. Pharmacol.*, **149**, 55–63.

To, L.P., Hunt, T.P. & Andersen, M.E. (1982) Mutagenicity of *trans*-anethole, estragole, eugenol, and safrole in the Ames *Salmonella typhimurium* assay. *Bull. Environ. Contam. Toxicol.*, **28**, 647–654.

Trubek Laboratories, Inc. (1958) Toxicological screening of eugenol, p-methoxybenzaldehyde and piperonal in rats. Class IX. Aromatic aldehydes. Unpublished report to the Flavor and Extract Manufacturers of the United States. Submitted to WHO by the Flavor and Extract Manufacturers Association of the United States, Washington DC, USA.

Weinberg, J.B., Rabinowitz, J.L., Zanger, M. & Gennaro, A.R. (1972) ^{14}C-Eugenol: I. Synthesis, polymerization, and use. *J. Dental Res.*, **51**, 1055–1061.

Woolverton, C., Fotos, P., Mokas, M. & Mermigas, M. (1986) Evaluation of eugenol for mutagenicity by the mouse micronucleus test. *Oral Pathol.*, **15**, 450–453.

Wooster, R., Ebner, T., Sutherland, L., Clarke, D. & Burchell, B. (1993) Drug and xenobiotic glucuronidation catalysed by cloned human liver UDP-glucuronosyltransferases stably expressed in tissue culture cell lines. *Toxicology*, **82**, 119–129.

Yoshimura, H., Nakamura, M. & Koeda, T. (1981) Mutagenicity-screening of anesthetics for fishes. *Mutat. Res.*, **90**, 119–124.

Young, S.S. (1987) Are there local room effects on hepatic tumors in male mice? An examination of the NTP eugenol study. *Fundam. Appl. Toxicol.*, **8**, 1–4.

Yuasa, A. (1974) Experimental studies on glucuronidation III. UDP-Glucuronosyltransferase activity and glucuronide excretion enhanced by oral administration of eugenol. *Jpn. J. Vet. Sci.*, **36**, 427–432.