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Review

Safety assessment of allylalkoxybenzene derivatives used as flavouring substances — methyl eugenol and estragole

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Summary

This publication is the seventh in a series of safety evaluations performed by the Expert Panel of the Flavor and Extract Manufacturers' Association (FEMA). In 1993, the Panel initiated a comprehensive program to re-evaluate the safety of more than 1700 GRAS flavouring substances under conditions of intended use. In this review, scientific data relevant to the safety evaluation of the allylalkoxybenzene derivatives methyl eugenol and estragole is critically evaluated by the FEMA Expert Panel. The hazard determination uses a mechanism-based approach in which production of the hepatotoxic sulfate conjugate of the 1′-hydroxy metabolite is used to interpret the pathological changes observed in different species of laboratory rodents in chronic and subchronic studies. In the risk evaluation, the effect of dose and metabolic activation on the production of the 1′-hydroxy metabolite in humans and laboratory animals is compared to assess the risk to humans from use of methyl eugenol and estragole as naturally occurring components of a traditional diet and as added flavouring substances. Both the qualitative and quantitative aspects of the molecular disposition of methyl eugenol and estragole and their associated toxicological sequelae have been relatively well defined from mammalian studies. Several studies have clearly established that the profiles of metabolism, metabolic activation, and covalent binding are dose dependent and that the relative importance diminishes markedly at low levels of exposure (i.e. these events are not linear with respect to dose). In particular, rodent studies show that these events are minimal probably in the dose range of 1–10 mg/kg body weight, which is approximately 100–1000 times the anticipated human exposure to these substances. For these reasons it is concluded that present exposure to methyl eugenol and estragole resulting from consumption of food, mainly spices and added as such,

Abbreviations: ABP, aminobiphenyl; ADI, Acceptable Daily Intake; AUC, area under the curve; bw, body weight; CYP450, cytochrome P450; DNA, deoxyribonucleic acid; EH, epoxide hydrolase; FAO/WHO, Food and Agriculture Organization of the United Nations/World Health Organization; FEMA, The Flavor and Extract Manufacturers Association; GRAS, Generally Recognized as Safe; GRASa, GRAS affirmed; GRASr, GRAS reaffirmed; GSH, glutathione; GST, glutathione *S*-transferase; GT, glucuronosyl transferase; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LDH, lactate dehydrogenase; LD_{50}, median lethal dose; MLA, mouse lymphoma cells; NAS, National Academy of Sciences; NHANES, National Health and Nutritional Examination Survey; NOAEL, no-observed-adverse-effect level; NTP, National Toxicology Program; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PCR-RFLP, polymerase chain reaction-restiction fragment length polymorphism; ppm, parts per million; SAL, *Salmonella typhimurium*; SCE, sister chromatid exchanges; SLR, Scientific Literature Review; UDS, unscheduled DNA synthesis.

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does not pose a significant cancer risk. Nevertheless, further studies are needed to define both the nature and implications of the dose–response curve in rats at low levels of exposure to methyl eugenol and estragole.

Keywords: Allylalkoxybenzenes; Methyl eugenol; Estragole; Hepatotoxicity; Carcinogenicity; Dose-related; National Toxicology Program; Generally recognised as safe (GRAS)

Contents

1. Explanation

1.1. Introduction

This review presents the key scientific data related to the safety of methyl eugenol and estragole, two structurally related *p*-allylalkoxybenzene derivatives, from consumption of traditional foods and from intake as flavouring substances. The two substances occur naturally in a variety of traditional foods, mainly in spices. They also occur in food as a result of the intentional addition of essential oils containing allylalkoxybenzene derivatives and from the intentional addition of the chemically identified substances. Estragole and methyl eugenol are common components of spices, mainly tarragon, basil, fennel, marjoram, mace, allspice, star anise and anise. Methyl eugenol and estragole are used as flavouring substances in baked goods, non-alcoholic beverages, condiments, and hard and soft candy at maximum levels of use less than 50 ppm (Hall and Oser, 1965). Methyl eugenol and estragole are consumed predominantly as components of traditional foods (Stofberg and Grundschober, 1987) (see section on Exposure).

In 2000, it was reported that chronic oral intake of high dose levels of methyl eugenol was associated with increased incidence of hepatotoxicity and liver and stomach neoplasms in F344/N rats and B6C3F1 mice (NTP, 2000). The results of this study together with recent data on the pharmacokinetics, metabolism, toxicity and genotoxicity for methyl eugenol, estragole and other allylalkoxybenzene derivatives are used here in a mechanism-based safety evaluation to interpret toxicity and related carcinogenic effects in laboratory rodents as they apply to their uses as flavourings by humans.

1.2. Regulatory status

Methyl eugenol and estragole are permitted for use as synthetic flavouring substances under conditions of intended use (21CFR Section 172.515). In 1965, the FEMA Expert Panel concluded that methyl eugenol and estragole are "generally recognized as safe" (GRAS) under conditions of intended use as flavouring

substances in food (Hall and Oser, 1965). In 1979, the Panel again evaluated the available data and affirmed the GRAS status of methyl eugenol and estragole for use as flavouring substances (GRASa). In 2001, the FEMA Expert Panel performed a third comprehensive review of all data relevant to the safety evaluation of methyl eugenol and estragole from use as flavouring substances in food. This document contains the review of the data and the Panel's interpretation of those data.

1.3. Structural classification

Chemically, estragole is 4-methoxyallylbenzene and methyl eugenol is 3,4-dimethoxyallylbenzene. The only structural difference between estragole and methyl eugenol is that the latter contains a second ring methoxy group. Since they contain the same skeletal structure and ring substituents, both substances are expected to exhibit similar metabolic fate, pharmacokinetics and toxicologic potential. By virtue of the fact that safrole is 3,4-methylenedioxyallylbenzene, it also contains many of the structural features (i.e. *O*-alkyl and *p*-allyl ring substituents) common to estragole and methyl eugenol. Therefore, key data on estragole, methyleugenol, safrole and other *p*-allylalkoxybenzene derivatives provide a more comprehensive chemical and biological basis upon which to evaluate the safety of these substances.

2. Biological data

2.1. Biochemistry

2.1.1. Absorption, distribution, biotransformation, and excretion

2.1.1.1. Introduction. Pharmacokinetic and metabolic information for methyl eugenol and estragole indicate that these substances undergo rapid and essentially complete absorption via the oral route (Sutton et al., 1985; Anthony et al., 1987). Metabolic pathways are regulated by dose. At low dose, ring substituents (e.g. methoxy) are metabolised and at high concentrations biotransformation switches additionally to the oxidation of the allyl side chain. As the dose is increased [0.05–1000 mg/kg body weight (bw)] in mice and rats, the extent of *O*-demethylation decreases while 1′-hydroxylation increases (Zangouras et al., 1981). The 1′-hydroxylation pathway is a significant metabolic activation pathway in mice and rats (Phillips et al., 1981; Swanson et al., 1981; Miller et al., 1983; Wiseman et al., 1985). Intoxication via the epoxidation of the allyl side chain is not as significant as activation via the 1′-hydroxylation pathway (Luo and Guenthner, 1995, 1996). Three metabolic options are available to methyl eugenol and estragole (see Fig. 1).

O-Demethylation (Pathway I, Scheme 1) of the *p*-methoxy substituent of estragole, the *m*- or *p*-methoxy substituent of methyl eugenol, or one of its metabolites (see below) yields the corresponding phenolic derivative, which may be excreted as the sulfate or glucuronic acid conjugate. This pathway is prevalent at low dose in humans and in rodents (Zangouras et al., 1981; Sangster et al., 1983, 1987; Anthony et al., 1987). Dose-dependent metabolism studies on the structurally related propenylalkoxybenzene derivative, 4-methoxypropenyl benzene (anethole), confirm that *O*-demethylation is the predominant metabolic pathway at low dose levels (< 10 mg/kg bw) in the rat and the mouse (Sangster et al., 1987).

Epoxidation of the side chain alkene yields the 2′,3′-epoxide (Pathway II, Scheme 1). The epoxide is detoxicated by epoxide hydrolase to form the diol and via glutathione conjugation (Luo and Guenthner, 1995). Although the epoxide does form DNA adducts in vitro, in vivo rapid detoxication by epoxide hydrolase (EH) and glutathione transferase (GST) prevents it from forming DNA adducts (Luo and Guenthner, 1996). The carboxylic acid formed via oxidation of the diol may be conjugated with glycine and excreted or undergo β-oxidation, cleavage and conjugation to the corresponding hippuric acid derivative.

1′-Hydroxylation to yield 1′-hydroxyestragole or 1′-hydroxymethyl eugenol is widely recognised as the primary intoxication pathway (Pathway III, Scheme 1) (Drinkwater et al., 1976; Zangouras et al., 1981; Miller et al., 1983). The 1′-hydroxy metabolite contains an unstable terminal alkene (i.e. 1′-hydroxy-2,3-alkene). To some extent, the 1′-hydroxy metabolite or its sulfate conjugate may be isomerized to yield the more stable 3′-hydroxy-1′,2′-alkene containing a readily oxidisable primary alcohol function. Oxidation of this alcohol produces a cinnamic acid derivative that may undergo β-oxidation and cleavage to yield a benzoic acid derivative. This metabolite may be excreted as the glycine conjugate (Solheim and Scheline, 1973; Delaforge et al., 1980).

Another option involves reaction of the labile sulfate conjugate of the 1′-hydroxy metabolite. This pathway is considered to produce the proximate hepatotoxic and hepatocarcinogenic agent in rodents. The unstable sulfate ester is anticipated to hydrolyse to form a reactive electrophilic intermediate (carbonium ion or quinonium cation) that binds hepatic proteins and hepatic DNA. The formation of protein and DNA adducts is dose dependent (Drinkwater et al., 1976; Swanson et al., 1981; Miller et al., 1982, 1983; Boberg et al., 1983; Gardner et al., 1995, 1996). Sulfate inhibition studies (Boberg et al., 1983) and in vivo–in vitro unscheduled DNA synthesis (UDS) assays of either estragole or methyl eugenol and their 1′-hydroxy metabolites (Caldwell et al., 1992; Chan and Caldwell, 1992) provide

additional evidence that the sulfate ester of the 1′-hydroxy metabolite is the principal intoxication metabolite in animals.

Additional biochemical studies have focused on the influence of dose and species on the formation of the 1′-hydroxy metabolite; the cytochrome P-450 (CYP-450) isoenzymes that catalyze the 1′-hydroxylation pathway, and the formation of protein and DNA adducts with the 1′-hydroxy metabolite. These studies are discussed in Section 2.1.1.4.

2.1.1.2. Pharmacokinetics. In male rats, greater than 95% of a single dose of 200 mg/kg body weight of methyl eugenol or 56–66% of a dose of 100 mg/kg body weight of estragole administered as a 1% suspension by stomach tube was excreted in the urine within 24 h (Solheim and Scheline, 1973). When the same dose levels were administered by intraperitoneal injection, greater than 85% of the methyl eugenol dose and 77–87% of the estragole dose was excreted in the urine after 24 h (Solheim and Scheline, 1973).

Greater than 71% of a 50 mg/kg bw oral dose of ^{14}C-methoxy-labelled estragole given to female rats was eliminated in the first 24 h with an additional 3.5% eliminated in the next 24 h. Approximately 1% remained in the carcass at 48 h. Approximately 38% was eliminated in the urine, 31% in expired air, and 1.3% in the faeces (Zangouras, 1982). In a dose-dependent toxicokinetic study, female Wistar rats were given dose levels of 0.05 to 1000 mg/kg bw of ^{14}C-estragole. At the low doses (0.05–50 mg/kg bw), the majority (55% on day 1 and 2.7% on day 2) of the dose was eliminated as ^{14}C-labeled CO_2 in expired air. Urinary elimination accounted for a total 32.5% of the total radioactivity after 2 days. At higher dose levels (500 and 1000 mg/kg bw), elimination of radioactivity via expired air was less (29% on day 1 and 17% on day 2) and urinary elimination was greater (30% on day 1 and 29% on day 2) indicating a changeover in metabolism and elimination (Anthony et al., 1987).

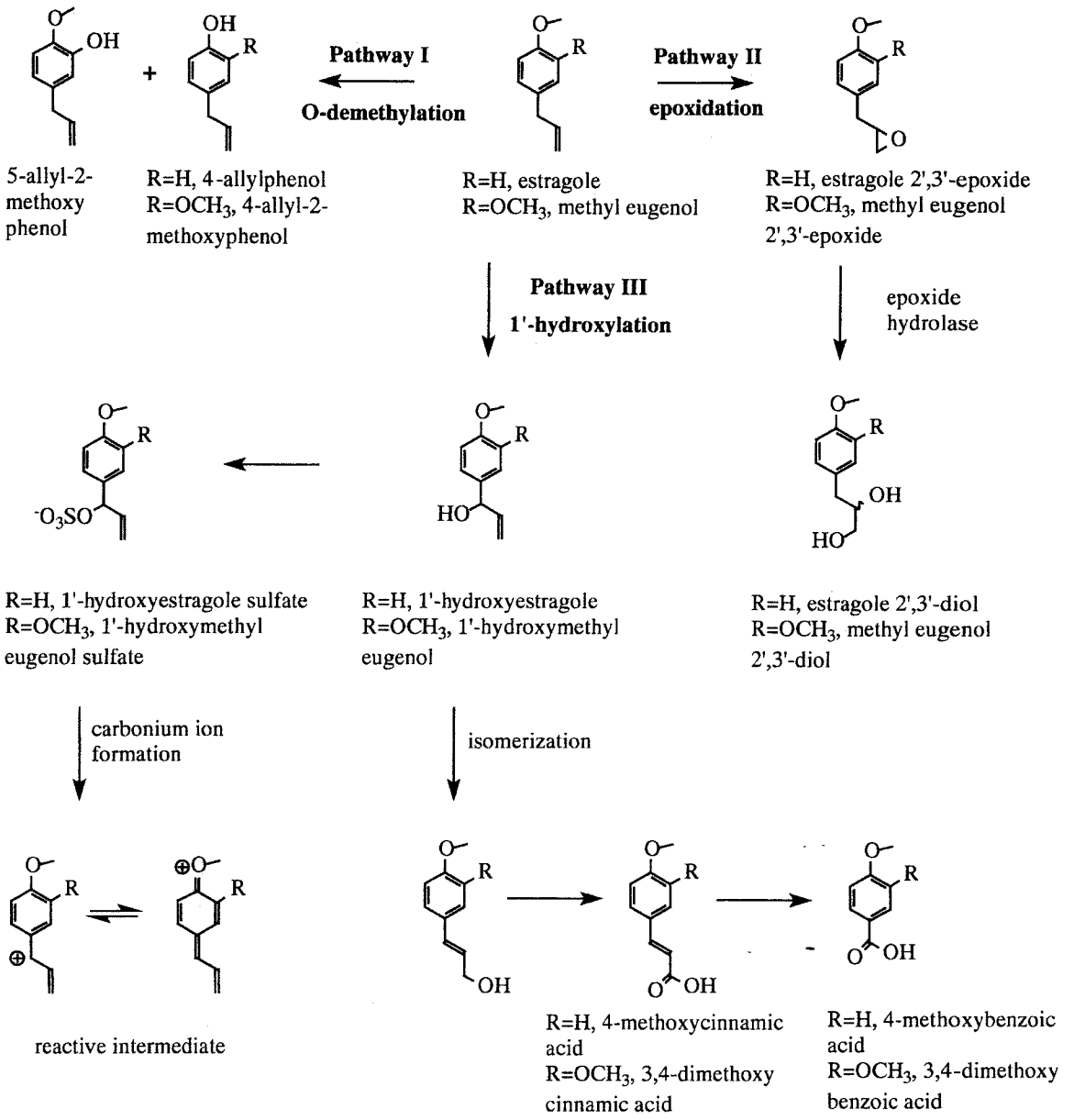
Peak plasma levels of 1.5 and 4 μg/ml were achieved when rats were given either a 37 or 150 mg/kg bw oral dose of methyl eugenol. Plasma half-lives for disappearance of methyl eugenol were 30–60 min and the area under the curve (AUC) was 97 and 225 μg/ml/min at 37 and 150 mg/kg bw, respectively (Graves and Runyon, 1995).

In a single-dose toxicokinetic study, F344/N rats (12/sex/group) were given 37 mg/kg bw by iv injection or 37, 75 or 150 mg/kg bw of methyl eugenol by oral intubation and blood was collected at time points up to 360 min (NTP, 2000). The iv and oral plasma concentration–time profiles were biphasic including an initial distribution phase followed by an elimination phase. Maximum plasma concentrations (C_{max}) of 0.656–3.84 μg/ml for males and 1.14 to 8.25 μg/ml for females were proportional to oral dose levels while time to maximum plasma levels (T_{max}) was rapid (5 min) and independent of dose. The AUC increased linearly with dose for both males and females. AUCs were in the range of 33.5–459 μg/ml min for males and 27–307 μg/ml min for females. Percent bioavailability also increased with dose. Bioavailability of methyl eugenol after a single oral dose was low (6% at 37 mg/kg bw and 19% at 150 and 300 mg/kg bw). Disappearance half-lives were in the range from 60 to 115 min for both sexes. In mice given 25, 50 or 75 mg/kg bw, peak plasma levels were similar to those for rats (0.38–3.10 μg/ml for males and 0.12–4.4 μg/ml for females) and were reached in 5 min (T_{max}) in all groups except females in the 25 mg/kg bw groups which showed T_{max} of 15 min. Plasma half-lives were shorter (30 min) and AUCs were significantly lower than those recorded for rats (4.91–48.4 for males and 3.27–60.5 μg/ml/min for females), indicating that methyl eugenol was eliminated more rapidly from the mouse. Seventy-two h after oral or iv administration of [^{14}C]methyl eugenol to male rats, radioactivity was concentrated mainly in the liver (liver:blood ratio, 2:3) (NTP, 2000).

In a second toxicokinetic study performed at the National Toxicology Program (NTP) (NTP, 2000), the pharmacokinetic profile was followed during repeated oral administration to rats and mice. Blood was taken from F344/N rats that had been treated with 37, 75, 150 or 300 mg/kg bw of methyl eugenol by gavage daily, 5 days per week for 6, 12 or 18 months. B6C3F1 mice treated at the same dose levels were monitored at 12 and 18 months. Absorption was extremely rapid in all dosed groups. Time to C_{max} was less than 5 min. Elimination from the blood was also rapid with elimination half lives of 1–2 h in both sexes.

At 6 months, peak plasma levels (C_{max}) increased with increasing dose for most groups. Female concentrations (1.4–2.4 μg/ml) were higher than males (0.5–0.4 μg/ml) at the two lowest doses, but male concentrations (1.3–4.0 μg/ml) were higher than those (0.8–3.1 μg/ml) of females at the two highest doses. Generally, at the same dose levels, C_{max} was lower after 6-months of daily exposure than after single-dose administration. Significant increases in both C_{max} and AUC between 6 and 12 months in the 150 and 300 mg/kg bw groups suggests that metabolic saturation is achieved during this time interval at higher dose levels. At all dose levels, females showed AUCs similar to naive animals while males at 37, 75 and 150 exhibited increased AUCs suggesting metabolic enzymatic induction plays a more important role in males. An increase in AUCs with time suggests a decrease in the capacity to metabolise methyl eugenol with age (NTP, 2000).

For mice given 35, 75 or 150 mg/kg bw per day for 2 years, absorption was also rapid. C_{max} was reached after 5 min and increased with increasing dose for both males and females. Elimination half-lives increased with dose



Scheme 1. Metabolism of allylalkoxybenzene derivatives in animals.

suggesting that the elimination was saturated for both sexes.

Male F344/N rats were given a single dose of 118 mg/kg bw [ring-^{14}C]methyl eugenol and blood and urine were collected regularly and analysed. Greater than 72% was eliminated in the urine, 13% in the faeces, and less than 0.1% in expired air after 72 h. Minute amounts (<0.4%) remained in the tissue at 72 h, with the majority being present in the liver. In female mice given the same dose, 85% was eliminated in the urine, 6% in the faeces, less than 0.1% in the expired air, and less than 0.3% in the tissue. The largest amount was found in the fat, followed by the muscle and liver (Burkey et al., 1999).

In humans (2), a 100 μg dose of ^{14}C-methoxy-labelled estragole given by gelatin capsule was eliminated in the urine with 35% eliminated after 8 h, 49.4% after 24 h, and 61.2% after 48 h. Greater than 11% was eliminated in expired air after 8 h. Approximately 70% of the dose was recovered within 48 h (Sangster et al., 1987).

In nine fasted human volunteers, ingestion of ginger snap cookies containing approximately 216 μg methyl eugenol (3.7 μg/kg bw) resulted in peak serum concentrations of 25–100 pg/g (approx. 0.000025–0.00010 μg/ml) with a mean of 16 pg/g (S. Masten, personal communication, 2000). In 209 US adults from an NHANES (National Health and Nutrition Examination Survey) III survey, serum methyl eugenol levels were in the range from non-detectable to 390 pg/g (ng/kg) with a mean of 24 pg/g, a 95th percentile of 78 pg/g, and a detection limit of 3.1 pg/g (Barr et al., 2000). Over

98% of those surveyed contained detectible levels of methyl eugenol.

Based on the above data, it may be concluded that estragole and methyl eugenol are rapidly absorbed by the oral route and metabolised in the liver. Compared with female rats, male rats are more prone to experience metabolic saturation after prolonged (>6 months), repeated exposure to high dose levels of methyl eugenol. Male rats also experience metabolic induction at lower dose levels and earlier in exposure than do female rats.

In rodents and in humans, routes of elimination at low dose include loss as carbon dioxide via expired air (i.e. arising from *O*-demethylation) and excretion of polar metabolites in the urine. At higher dose levels the fraction eliminated by expired air decreases while the fraction of non-volatile urinary metabolites increases.

2.1.1.3. Metabolism. In rats, a 100 mg/kg bw dose of estragole given by the oral or ip route was excreted in the urine mainly as the *O*-demethylation product (4-allylphenol (39% oral or 46% ip) within 48 h (see Fig. 1). Other metabolites accounting for 17% of the oral dose or 31% of the ip dose included the product of epoxidation, hydration and subsequent oxidation of the terminal alcohol (3-hydroxy-3-(4-methyoxyphenyl) propionic acid) of the allyl side-chain and the products of alkene isomerisation, oxidation of the resulting C_3 position, and beta-oxidation yielding 4-methoxybenzoic acid and 4-methoxyhippuric acid. Approximately 5–10% of the dose was excreted as the 1′-hydroxylation metabolite, 1′-hydroxyestragole (Solheim and Scheline, 1973).

Oxidation of the 3,4-methylenedioxyphenyl moiety is the major metabolic pathway in mammals given safrole (Kamienski and Casida, 1970). Safrole was largely metabolised by oxidation of the methylene group in male Swiss–Webster mice, Sprague–Dawley rats, or hamsters administered ^{14}C-labeled methylenedioxy-4-allylbenzene (safrole). The radiolabel ultimately appeared as labeled formate arising as the final oxidation product.

A single ip injection of 200 mg/kg bw of estragole, methyl eugenol or safrole was given to male Wistar rats and urine was collected every 2 h for 24 h. Twenty-four hours after treatment, animals were terminated and the livers were removed. Urinary metabolites included the epoxide of the parent substance and dealkylated safrole epoxide and the *O*-demethylated metabolites of methyl eugenol (allylcatechol epoxide) and estragole (allylphenol epoxide). Liver homogenates showed the presence of

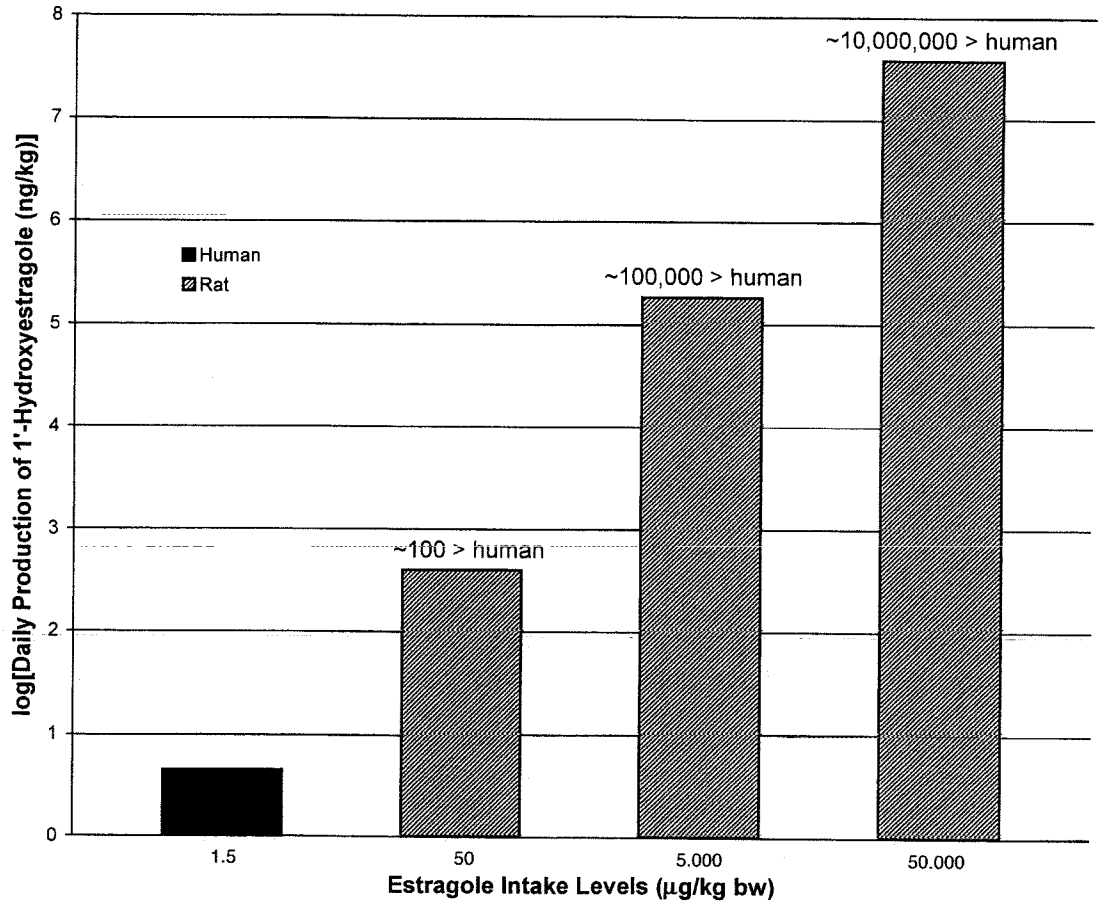


Fig. 1. Dose-dependent exposure to 1′-hydroxyestragole: daily urinary excretion of active metabolite by humans and rats given estragole.

safrole epoxide metabolites but not those of methyl eugenol or estragole. Liver microsomal preparations show the presence of the epoxide metabolite identified in the urine for all three substances (Delaforge et al., 1980).

Twenty-one-day-old mice were given 185 μmol/100 g bodyweight of either estragole or safrole by ip injection and the urine was analysed for 1′-hydroxy metabolites 24 h later. The dose level corresponds to 274 mg/kg bw of estragole and 300 mg/kg bw of safrole. Approximately 23% of estragole and 12% of the safrole was recovered from the 24-h urine as the corresponding 1′-hydroxy metabolite, whereas adult male mice (9–12 weeks) excreted up to 46% of the 300 mg/kg bw ip dose of safrole as 1′-hydroxysafrole (Drinkwater et al., 1976).

Formation of the 1′-hydroxy metabolite has been shown to be dose dependent in both mice and rats (Zangouras et al., 1981). When [^{14}C-methoxy]estragole was administered to rats (orally) and mice (ip) at dose levels of 0.05, 5, 500 or 1000 mg/kg bw, the proportion of the dose excreted in the urine as the glucuronide of 1′-hydroxyestragole increased with dose. Only 0.9% of the dose was excreted in the urine of rats given 0.05 mg/kg bw while 8.0% was found at 1000 mg/kg bw. The total production and exposure to the 1′-hydroxy metabolite increased significantly (1224–255,000 nmol/kg/day) as the dose was increased from 5 to 500 mg/kg. Conversely, the same increase in dose resulted in a decrease in *O*-demethylation from approximately 40–20% in both mice and rats. Thus, an increase in dose and a shift in metabolic pathways produces a marked increase in exposure to the 1′-hydroxy metabolite.

Evidently, at low dose, the 1′-hydroxylation pathway is a minor pathway for the metabolism of allylalkoxybenzene derivatives also in humans. In a study on the fate of [methoxy-^{14}C]estragole in humans, two male volunteers were fed a gelatin capsule containing 100 μg [methoxy-^{14}C]estragole (1.5 μg/kg bw). The bulk (72 and 67%) of the radioactivity was accounted for in the urine and as exhaled CO_2 within 48 h. Principal identified metabolites included those derived from *O*-demethylation and oxidative degradation of the allyl side chain (i.e. 4-methyoxyhippuric acid, the glycine conjugate of 4-methoxycinnamic acid, and 4-methoxyphenyllactic acid). Urinary 1′-hydroxyestragole accounted for an average of 0.3% of the total dose (Sangster et al., 1987). The dose administered (1.5 μg/kg bw/day) is within the range of estimated daily human exposure (1–10 μg/kg bw/day) (see Section 4.2).

The urine of eight human volunteers given single oral doses of either 0.163 or 1.655 mg safrole contained metabolites formed by oxidation of the methylenedioxy group, 1,2-dihydroxy-4-allylbenzene and 1-hydroxy-2-methoxy-4-allylbenzene. 1′-Hydroxysafrole was not detected (Benedetti et al., 1977). The importance of metabolism of the aromatic alkoxy substituent at low dose levels in humans has also been observed in structurally related 4-propenylmethoxybenzene (anethole) (Sangster et al., 1987; Caldwell and Sutton, 1988; Newberne et al., 1999).

Studies of the cytochrome P450 (CYP-450) enzyme induction of the 1′-hydroxylation pathway in rat and human liver microsomes indicate that the reaction is catalysed predominantly by CYP2E1 and probably CYP2C6. The rate of 1′-hydroxylation of methyl eugenol varied widely in 13 human liver microsome samples (37-fold), but the highest activities were similar to the activities in control rat liver microsomes (Gardner et al., 1997). At low substrate concentrations, in control rat liver microsomes, 1′-hydroxylation of methyl eugenol was induced by phenobarbital, isosafrole and dexamethasone, but significantly inhibited by diallylsulfide (40%), α-naphthoflavone (25%) and *p*-nitrophenol (55%).

A marked increase in methyl eugenol–protein adducts occurred when CYP-450 was induced by an assortment of CYP-450 inducers including dexamethasone. Autoinduction of the 1′-hydroxylation pathway was reported in hepatic microsomes of rats given 30–300 mg methyl eugenol/kg bw/day orally for 5 days but not in rats given 10 mg/kg bw/day for 5 days (Gardner et al., 1997).

It appears that the 1′-hydroxylation pathway is more prominent at higher levels of exposure (i.e. >10 mg/kg bw). Certainly at low dose (100 μg or 1.5 μg/kg bw), human production of 1′-hydroxy metabolite is expected to be very low given that urinary excretion of the 1′-hydroxy metabolite is <0.5%. *O*-Demethylation is an important pathway at low dose. At low dose levels, humans, mice and rats show a similar tendency to metabolise allylalkoxybenzene derivatives by *O*-demethylation. At low dose significant amounts of estragole or methyl eugenol are *O*-demethylated, but as dose levels increase 1′-hydroxylation and epoxidation of allylalkoxybenzene derivatives increase. The total daily urinary production of the 1′-hydroxy metabolite increases significantly, as much as 6000 times, as the dose increases from 50 μg/kg bw to 50 mg/kg bw (a 1000-fold increase) and the metabolism shifts to the CYP450 catalysed 1′-hydroxylation pathway (roughly a six-fold increase) (Zangouras et al., 1981; Anthony et al., 1987).

In summary, metabolic activation in mice and rats maintained on high dose levels in the NTP study resulted in a marked increase in production of 1′-hydroxy methyl eugenol. Increased 1′-hydroxylation of estragole or methyl eugenol is reflected by dose-dependent increases in urinary levels of this metabolite (<1.0% at 0.05 mg estragole/kg bw, 3.6% at 5 mg/kg bw and 7.6% at 500 mg/kg bw) (Zangouras et al., 1981). The lowest gavage dose level of 37.4 mg/kg bw/day given to rats in the NTP study is calculated to provide a daily urinary output of approximately 2 mg/kg bw of the 1′-hydroxy

metabolite, while at 0.05 mg/kg bw urinary output is only 0.00045 mg/kg bw which is approximately a six-fold decrease when compared to the predicted output of 0.0027 mg/kg bw.

2.1.1.4. Protein and DNA adduct formation and related biochemistry studies. Based on the evidence that the hepatotoxicity and hepatocarcinogenicity of estragole and methyl eugenol in rodents have been related to the formation of the corresponding 1′-hydroxy metabolite (see below), the induction of tumours may also be dose related. In repeated oral dose studies in rats, low doses (10 or 30 mg/kg/day for 5 days) of methyl eugenol have been shown to produce a single 44 kDa microsomal protein adduct which is likely formed from the reaction of the electrophilic 1′-hydroxylation metabolite (carbonium ion) with a peripheral membrane protein, possibly the CYP450 enzyme catalysing the hydroxylation reaction. It is also the major adduct at higher dose levels (100 and 300 mg/kg/day) which have been shown to produce as many as 20 other protein adducts. The formation of protein adducts has been directly related to the formation of the 1′-hydroxy metabolite (Borchert et al., 1973; Gardner et al., 1995) in a dose-related manner (Gardner et al., 1995). A similar pattern of adduct formation occurs in vitro when the 1′-hydroxy metabolite was incubated with rat hepatocytes (Gardner et al., 1995, 1996).

In studies beginning two decades ago, methyl eugenol and estragole, the 1′-hydroxy metabolites of methyl eugenol and estragole, and the corresponding sulfate esters of the 1′-hydroxy metabolites were shown to form DNA adducts in vivo and in vitro. Adult female CD-1 mice (mean weight 35 g) were given 12 μmol/mouse (58 mg/kg) of [2′,3′-^{3}H]1′-hydroxyestragole by ip injection in trioctanoin and DNA adduct formation monitored over 20 days post-exposure. Similarly, 9-day-old male or female B6C3F1 mice (mean weight, 6 g) were given ip injections of 0.5 μmol (14 mg/kg) of labelled estragole and sacrificed after 23 h. Three adducts were formed by the reaction of 1′ or 3′ positions (*cis* or *trans* isomers) of estragole with the exocyclic amino group (N^2) of deoxyguanosine. An additional adduct was formed by the reaction of the 3′ position of estragole and the (N^6) position of deoxyadenosine. Unlike adducts of aromatic amines (e.g. *N*-acetyl-2-aminofluorene) which persist at near-maximum levels of binding for several weeks, the three adducts of estragole–deoxyribonucleoside were removed rapidly from mouse liver DNA. Timed measurement of DNA adducts indicated a biphasic loss indicated by a sharp decline in one of the two major 1′-hydroxyestragole adducts followed by relatively constant levels of liver DNA adducts from days 3–20. This suggests that at least one of the adducts undergoes excision repair. Dose levels of the 1′-hydroxyestragole in the adult female and preweanling male and female mice were approximately 58 mg/kg bw and 14 mg/kg bw, respectively (Phillips et al., 1981).

In ^{32}P-post-labelling experiments with adult female CD-1 mice (mean weight, 25 g) (Randerath et al., 1984), a 2 or 10 mg dose of estragole, methyl eugenol, safrole or other alkenylbenzene derivative was given by ip injection and liver DNA samples were collected 24 h later. The dose levels in this study were equivalent to 100 or 500 mg/kg bw of each test substances. Safrole, methyl eugenol and estragole show binding activities higher than allylbenzene, anethole and other allyl substituted benzene derivatives. Similar to the previous experiment, a rapid drop in total adduct formation occurred within 7 days after dosing and was followed by a relatively constant level over the next 140 days. The authors noted that the significant decrease in DNA adduct levels was probably related to DNA repair processes.

In a related ^{32}P-post-labelling experiment (Phillips et al., 1984), newborn male B6C3F1 mice were given 0.25, 0.5, 1.0 and 3.0 μmol of the same series of allylbenzene derivatives by ip injection on days 1, 8, 15 and 22, respectively, after birth. Dose levels on days 1 and 22 were estimated to be approximately 27 and 35 mg/kg bw, 1′-hydroxyestragole and 1′-hydroxysafrole, respectively. Mice were terminated on days 23, 29 and 43 and their liver DNA was isolated and analysed. Highest DNA adduct levels were measured for methyl eugenol, estragole and safrole. A significant ($P<0.05$) amount of adduct was detected at 43 days. Based on the results of a study of carcinogenic activity of these substances in the same species and strain (Miller et al., 1983), the authors concluded that adduct levels of at least 15 pmol/mg of DNA at 23 days were required for statistically significant tumour formation (Phillips et al., 1984). The authors also noted that, compared with adults, newborn mice showed greater sensitivity to alkenylbenzene carcinogenicity.

In a study of the reversible and irreversible inhibition of isoenzymes of glutathione transferase (GST) by allylalkoxybenzene derivatives, purified α-, μ- and π-class isoenzymes of (GST) (10–30 nM) prepared from rat and human liver were incubated with different concentrations of methyl eugenol and other allylhydroxy- and allylmethoxy-benzene derivatives. Immediately after incubation, enzyme activity for conjugation was measured using 1-chloro, 2,4-dinitrobenzene as a substrate. In rat liver, all classes of GST isoenzymes were most strongly inhibited by methyl eugenol, whereas in human liver GST, inhibition capacity was similar for all allylbenzene derivatives (Rompelberg et al., 1996).

Based on the protein and DNA adduct studies it is concluded that methyl eugenol and estragole can form covalently-bound protein and DNA adducts. It is anticipated that the 1′-hydroxy derivative is the reactive intermediary metabolite that forms protein and DNA adducts, since the 1′-hydroxy metabolite forms these

same adducts but at lower dose levels than for the parent substance. Studies performed at relatively high dose levels (15 mg/kg bw) demonstrate that the 1′-hydroxy metabolite reacts with an exocyclic amine function to form a single covalent bond to the deoxyribonucleoside. Clearly, at high dose levels, DNA adduct formation has been directly related to administration of the parent allylalkoxybenzene derivative or its principal hepatotoxic metabolite. Currently there is no information on the formation of DNA adducts at dose levels less than 10 mg/kg bw; there is some evidence that liver tumours are not induced at a low dose level (1.5 mg/kg bw) of 1′-hydroxy metabolite (Wiseman et al., 1987) (see Section 2.2.2.2).

2.2. Toxicology

2.2.1. Short-term studies

2.2.1.1. 91-Day dietary study with methyl eugenol in rats. In a 91-day feeding study of methyl eugenol, groups of SD rats (24/sex) were maintained on diets resulting in an average daily intake of 18 mg/kg bw. Weekly measurements of body weight and food consumption revealed no significant differences between test and control animals. Haematological examinations, blood chemical determinations and urine analysis monitored at weeks 6 and 12 revealed normal values. At necropsy, organ weight measurements indicated a small but significant ($P<0.05$) increase in relative liver weight for male rats only. Histopathological examination of 27 major organs and tissues revealed no alterations that could be associated to administration of the test material (Osborne et al., 1981).

2.2.1.2. 90-Day gavage study with methyl eugenol in mice and rats. Groups of male (10) and female (10) F344 rats and B6C3F1 mice were administered 0, 10, 30, 100, 300 or 1000 mg/kg bw of methyl eugenol in 0.5% methylcellulose daily by gavage, 5 days a week for 14 weeks. The final mean body weight gains of male rats receiving 300 and 1000 mg/kg bw/day and all the dosed female rats were significantly ($P=0.01$) less than those of the vehicle control. Liver weights in male rats dosed with 100 mg/kg bw per day or greater and in female rats dosed with 300 mg/kg bw/day or greater were significantly higher than those in control rats. Relative liver weights of male rats at 30 mg/kg bw per day were increased (14.08 g) compared with the vehicle controls (12.87 g) but not with respect to untreated controls (13.56 g). A significant increase in testis weight was observed in male rats receiving 1000 mg/kg/day. Haematological examination revealed a decreased mean packed red cell volume in 300 mg/kg/day male rats and in male and female rats receiving 1000 mg/kg/day. There were also increased platelet counts and increased alanine aminotransferase and sorbitol dehydrogenase activities in male and female rats receiving 100 mg/kg/day or greater. Additionally, hypoproteinemia, hypoalbuminemia and increased bile acid concentrations were evident in male and female rats receiving 300 mg/kg/day or greater. An increase in the incidence of adrenal gland cortical hypertrophy and/or cytoplasmic alteration in the submandibular gland occurred in 100 mg/kg or greater male and female rats. The incidences of atrophy and chronic inflammation (chronic gastritis) of the glandular stomach mucosa were significantly increased in male and female rats administered 300 mg/kg or greater, and there was a hepatocellular adenoma in one male rat administered 1000 mg/kg. There were no significant findings at 10 mg/kg bw/day (NTP, 2000).

In mice, low survival rates were reported at the highest dose level of methyl eugenol in males and females. Mean body weight gains of male and female mice given 300 mg/kg were significantly less than those of the vehicle control. There was a statistical increase ($P<0.05$) in liver weights in male mice dosed with 30 mg/kg bw/day or greater and in female mice dosed with 300 mg/kg bw/day compared with those of the respective control groups. Increased incidences of cytologic alteration, necrosis, bile duct hyperplasia and subacute inflammation were observed in the liver of 1000 mg/kg male mice and 300 mg/kg and greater female mice. A significant increase in testis weight was observed in male mice receiving 100 or 300 mg/kg/day. There were no significant findings at 10 mg/kg bw/day (NTP, 2000).

2.2.2. Long-term studies

2.2.2.1. Short- and long-term dietary studies with safrole in rats. As early as 1965, long-term studies in laboratory rodents indicated that allylalkoxybenzene derivatives were hepatocarcinogenic when administered in the diet, often at levels that caused liver and gastric damage. Groups (10/sex/group) of male and female Osborne–Mendel rats were maintained on diets containing 0 (control), 10,000 ppm for 62 weeks, 5000 ppm (25 M & 25 F), 2500 or 1000 ppm safrole daily for 2 years. The levels are calculated to provide an average daily intake of approximately 500, 250, 125 or 50 mg/kg bw/day, respectively. Diets were prepared fresh weekly. Measurement of body weight, food intake and general condition were made weekly. Haematological examinations were performed at 3, 6, 12, 22 months and at termination of the study. At the highest dietary level, all rats were dead at 62 weeks. Measurement of body weight and food intake showed reduced body weight gain at the three highest dose levels in males and females and in females provided the 1000 ppm diet.

At the highest dose, the liver was enlarged, with mottling and irregular single and multiple tumour masses. Microscopic examination revealed hepatocyte enlargement and steatosis, nodules showing cystic necrosis, cirrhosis, adenomatoid hyperplasia and hepatocellular adenomas

and carcinomas. Other findings included atrophy and atypical regeneration of the mucosal glands of the stomach with associated fibrosis and hyalinisation of the surrounding stroma. Atrophy of the testes was also reported. At 5000 ppm, there was increased mortality in males. Liver changes were of the same type and severity as in the 10,000 ppm group. A statistically significant increase in malignant primary hepatic tumours was reported. Other effects included a slight increase in chronic nephritis in females and mild hyperplasia of the thyroid. At 2500 ppm, moderate liver damage was reported without cirrhosis or tumours. A moderate increase in the incidence of nephritis was reported. At the 1000 ppm level, liver damage was slight. No malignant tumours or cirrhosis were observed (Hagan et al., 1967).

In another study using the same protocol, male and female Osborne–Mendel rats (25 M & 25 F) were maintained on diets containing safrole at concentrations of 0 (control), 100, 500, 1000 or 5000 ppm for 2 years. The levels are calculated to provide an average daily intake of approximately 0, 5, 25, 50 or 100 mg/kg bw/day, respectively. At the highest dose, reduced body weight gain was reported in both sexes. Haematological examinations revealed mild anemia and leucocytosis. At dietary levels of 5000 ppm, there was a statistically significant increase in benign and malignant neoplasms of the liver. At the 1000 ppm level, there was slight to moderate liver damage but no evidence of malignant liver neoplasms or cirrhosis. At 500 and 100 ppm, there was slight liver damage but no evidence of malignant hepatocellular carcinomas or cirrhosis after 2 years (Long and Jenner, 1963).

2.2.2.2. Carcinogenicity studies with estragole, 1′-hydroxyestragole and estragole epoxide in mice. In a multipart study evaluating the carcinogenic potential of allylalkoxybenzene derivatives, groups of CD-1 female mice (mean weight 24 g) were maintained on a diet containing 2300 or 4600 ppm estragole or 2500 ppm 1′-hydroxy estragole. The authors estimated that the dietary levels corresponded to an average daily intake of 150–300 and 300–600 mg/kg bw for animals on the 2300 and 4600 ppm estragole diet, respectively, and 180–360 mg/kg bw for animals on the 1′-hydroxyestragole diet. To avoid intolerance the dietary concentration was reduced by 75% for the first 10 days and 50% for the next 10 days. The target diet was then maintained for 12 months. Survival at 20 months was slightly lower (68–70%) for estragole fed animals compared to control animals (78%). The average lifespan of mice given 1′-hydroxyestagole was 13.6 months compared to 18 months in controls. Body weights measured at 1, 4 and 8 months were markedly reduced at 4 and 8 months compared with controls. At 10 months, the incidence of hepatomas was 58% for animals at 2300 ppm estragole, 71% for animals at 4600 ppm estragole and 56% for animals at 2500 ppm of 1′-hydroxyestragole and 0% in controls. Histopathological examinations revealed portal fibrosis, chronic inflammation and bile duct proliferation in addition to the tumours. Varied number of ceroid-laden histocytes and focal area of hyperplasia and megalocytosis were also reported. Four mice fed 4600 ppm estragole had hepatic angiosarcomas (Miller et al., 1983).

In another part of the study, male (55) and female (49) CD-1 mice were administered 370 mg/kg of estragole by gavage twice a week for 10 doses beginning at 4 days of age. The mice were weaned at 35 days of age. Hepatomas were observed as early as 11 months. At 14 months, 73% of the males (3.5 hepatomas/mouse) and 24% of control males (0.6 hepatomas/mouse) exhibited hepatomas. The incidence of hepatomas in females (9%, 0.1 hepatomas/mouse) was not statistically different from control females (2%, 0.02 hepatomas/mouse) (Miller et al., 1983).

Male (50) and female (50) CD-1 mice were administered a total dose of 9.45 μmol/mouse of estragole or estragole epoxide or 1.87 μmol/mouse of 1′-hydroxyestragole by ip injection distributed in a ratio of 1:2:4:8 on days 1, 8, 15 and 22, respectively, of life. These doses correspond to 0.63, 1.26, 2.52 and 5.04 μmol/mouse, respectively. The mice were weaned at 22 days of age. At 12 months, 65% of the mice receiving estragole exhibited hepatomas (1.7 hepatomas/mouse) vs 26% of controls (0.5 hepatomas/mouse) exhibited hepatomas. The incidence of hepatomas in mice given estragole epoxide (40%, 0.6 hepatomas/mouse) was not statistically different from control (26%, 0.5 hepatomas/mouse). For 1′-hydroxyestragole, 93% of the mice receiving the test substance (2.7 hepatomas/mouse) and 15% of control males (0.2 hepatomas/mouse) exhibited hepatomas (Miller et al., 1983).

2.2.2.3. Carcinogenicity studies with 1′-hydroxyestragole and 1′hydroxysafrole in different strains of mice. In a study using a hybrid strain of B6C3F1 mice, and the parent strain, C3H/He male and female mice and C57BL/6 male and female mice, the mice were given ip injections of 1′-hydroxyestragole on days 1, 8, 15 and 22. Dose levels were 0.1 μmol on day 1, 0.04 μmol on days 8 and 15, and 0.08 μmol on day 22 after birth. The levels are calculated to provide 11.7 on day 1, 18.8 on day 8, 9.3 on day 15 and 10.1 mg/kg bw on day 22, respectively. The experiment was terminated after 14 months. The first tumour-bearing mouse was observed at 10 months. At 12 months, 76% of the treated C3H/He male mice (3.0 hepatomas/mouse) and 26% of control mice (0.3 hepatomas/mouse) exhibited hepatomas. The incidence of hepatomas in C3H/He female mice (6% 0.06 hepatomas/mouse) was not statistically different from those of control females. For C57BL/6 mice, the incidence of hepatomas in treated males was 14% (0.3 hepatomas/mouse) and was 5% (0.07 hepatomas/mouse) in control males. No hepatomas were observed

in treated or control B57BL/6 female mice (Wiseman et al., 1987).

In another part of the study, groups of male B6C3F1 mice were given single ip injections of 0.10 μmol/g (15 mg/kg) of body weight of 1′-hydroxyestragole or 1′-hydroxysafrole 12 days after birth. Animals were sacrificed after 12 months and incidence of hepatic tumours were measured. A second group of males was given a lower dose of 0.01 μmol/g of body weight. A statistically significant increase in the incidence of hepatomas/mouse were observed for both substances at 0.1 μmol/g bw, but no significant increase was observed at the low dose of 0.01 μmol/g bw (1.5 mg/kg) (Wiseman et al., 1987).

2.2.2.4. 2-Year bioassay with methyl eugenol in rats and mice by the National Toxicology Program (NTP). Male and female F344/N rats were administered methyl eugenol in 0.5% methylcellulose by gavage daily at dose levels of 37, 75 or 150 mg/kg bw per day, 5 days per week for 2 years (NTP, 2000). Stop-exposure groups received 300 mg/kg doses for 53 weeks followed by the vehicle only (0.5% methylcellulose) for the duration of the study.

All 150 and 300 mg/kg males died before the end of the study. Mean body weights of all dosed groups were less than those of the vehicle controls throughout the study. The incidences of liver non-neoplastic lesions in dosed groups of male and females were increased at 6 months, 12 months and 2 years. There were statistically significant increases in oval cell hyperplasia, hepatocyte hypertrophy and eosinophilic foci at all dose levels in male and female rats. At the three highest doses (75, 150 and 300 mg/kg bw per day) atypical focal bile duct hyperplasia, focal cystic degeneration and mixed cell foci were observed, more in males than females. Many of the same non-neoplastic lesions of the liver were reported in the 300 mg/kg bw groups of male and female rats at both 6 and 12 months in the stop-exposure group. Non-neoplastic lesions of the glandular stomach included statistically significant increases in mucosal atrophy at all dose levels and neuroendocrine hyperplasia at the three highest dose levels in females and at all dose levels in males. There was a significant increase in the incidence of nephropathy in females at 300 mg/kg, and the incidence of renal tubule hyperplasia was higher in the 75 mg/kg or greater groups than in the vehicle control.

Methyl eugenol-related liver neoplasms occurred in all dosed groups and comprised hepatocellular adenomas and carcinomas, hepatocholangiomas, and hepatocholangiocarcinomas. There was a statistically significant increase ($P=0.049$ in males and $P=0.017$ in females at 37 mg/kg bw; $P<0.001$ for all other treated groups) in the incidence of hepatocellular adenomas and carcinomas in all dose groups of males and female rats. Hepatocholangiomas and hepatocholangiocarcinomas were reported in the 150 mg/kg bw group of males (2/50, 4%) and females (3/49, 6%) and at higher incidence in the 300 mg/kg bw stop-exposure groups of males (13/50, 26%) and females (17/50, 34%). The appearance of cholangiocarcinomas and bile duct dysplasia was said to provide *some* additional evidence of carcinogenicity based on the rarity of these lesions in F344/N rats (historical incidence, 3/2145, 0.1%).

Both benign (3/50, 6%) and malignant (4/50, 8%) neuroendocrine cell neoplasms of the glandular stomach were reported in males at 150 mg/kg bw and in the 300 mg/kg bw stop-exposure group (2/49, 4.1% benign and 2/49, 4.1% malignant). The incidence of these neoplasms was much higher in females at dose levels of 75 mg/kg bw (13/50, 26% benign and 12/50, 24% malignant) and greater.

There were also significant increases in the incidence of: malignant mesothelioma in male rats given more than 150 mg/kg; and of mammary gland fibroadenoma in 75 and 150 mg/kg males; and fibroma of the subcutaneous tissue in 37 and 75 mg/kg males. These neoplasms were not found in female rats at any dose level.

In female mice and, to a lesser extent, in male mice there was evidence of hepatotoxicity of methyl eugenol. Significant increases in oval cell hyperplasia, eosinophilic foci, hepatocyte hypertrophy and necrosis, hematopoietic cell proliferation, hemosiderin pigmentation and bile duct cysts were observed at all dose levels in male and female mice. Non-neoplastic lesions of the glandular stomach included statistically significant increases in hyperplasia, ectasia, atrophy at all dose levels in both males and females and mineralisation and necrosis in lower incidence also in both sexes incidences of chronic atrophic gastritis was high. Gastric tumours were found in two high-dose males. The incidence of hepatocellular adenomas, hepatocellular carcinomas and hepatoblastomas was high in both treated and control male and female mice. While control males and females showed tumour rates of 63% (31/49) and 50% (25/50), respectively, and all treatment groups of males and females had tumour rates in excess of 92% with the exception of high-dose male rates in which the tumour rate was 82% (41/50). Evidence of infection by *Helicobacter hepaticus* was found by PCR-RFLP, but associated hepatitis was not found.

Because of the evidence of toxicity of methyl eugenol in all groups of rats and mice, the study cannot be recognised as conclusive for carcinogenicity at lower, non-toxic doses. In particular, the hepatic damage undoubtedly altered the metabolism of the compound, and the gastric damage probably altered its absorption.

2.2.3. Genotoxicity studies for allylalkoxybenzene derivatives and their 1′-hydroxy and 2,3-epoxide metabolites

The literature on the genetic toxicology of *p*-alkoxyallylbenzene derivatives, estragole (4-methoxyallylbenzene) and methyl eugenol (3,4-dimethoxyallylbenzene)

is fairly extensive, especially with regard to the results of in vitro assays. Additionally, both compounds yield similar results, as might be expected based on their structural similarity. Methyl eugenol was negative in multiple tests in various strains of *Salmonella typhimurium* and *Saccharomyces cerevisae* with and without metabolic activation (Dorange et al., 1977; Sekizawa and Shibamoto, 1982; Mortelmans et al., 1986; Schiestl et al., 1989; Brennan et al., 1996). Estragole was also negative in common strains of *S. typhimurium* with and without metabolic activation (Dorange et al., 1977; Sekizawa and Shibamoto, 1982; To et al., 1982; Zeiger et al., 1987; Zani et al., 1991).

In one study (To et al., 1982), a significant increase in the revertants per plate was reported for strain TA1538 in the presence of S-9 and PAPS (3′-phosphoadenosine 5′-phosphosulfate) cofactor. The authors proposed that mutagenic response was related to the formation of the sulfate ester of an active metabolite. All other strains of *S. typhimurium* were not mutagenic in assays using PAPS.

Results of in vitro Ames assays with the metabolites of estragole have given equivocal results. The 2,3-epoxide of estragole and 1′-hydroxyestragole were positive in strains TA100 and TA1535, but negative in TA98 with or without S-13 metabolic activation (Swanson et al., 1979). But in a different study no evidence of mutagenicity was reported when 1′-hydroxyestragole was incubated with strains TA98 and TA100 of *S. typhimurium* with and without S-13 metabolic activation. Addition of PAPS as a cofactor did not induce an increase in revertants. 1′-Acetoxyestragole was mutagenic in strains TA98 and TA100 but not in a dose-dependent manner (Drinkwater et al., 1976). Overall, the alkoxyallylbenzene derivatives do not appear to be mutagenic in *S. typhimurium*.

Estragole concentrations of 10^{-3}–10^{-5} M did not induce the formation of chromosomal aberrations in V79 cells with and without metabolic activation or in primary rat hepatocytes (Muller et al., 1994). Methyl eugenol produced sister chromatid exchange (SCE) in Chinese hamster ovary cells only in the presence of metabolic activation and at near cytotoxic levels. Therefore, the positive findings likely occurred secondary to cytotoxicity in which release of lysosomal nucleases may have resulted in a false positive response. When administered by gavage at doses up to 1000 mg/kg during a 14-week study, methyl eugenol was negative

Table 1
Oral human intake of methyl eugenol from food[a], essential oils[b] and flavour substances[c]

Food	Volatile oil in plants (%)	Mean concentration of ME in oil	1999 Reported annual volume (lb)	Intake (lb/year)	Intake (kg/year)	Intake (kg/p/d)	Intake (μg/p/d)	Intake (μg/kg bw/d)
Anise	0.2	0.0001	518112	0.1	0.05	4.9E-13	0.00	0.00001
Basil (dried)	2.1	0.026	3610861	1971.5	887	9.3E-09	9.35	0.156
Basil (fresh)	0.3	0.0011	361086	1.2	1	5.7E-12	0.01	0.0001
Mace	13	0.002	65611	17.1	8	8.1E-11	0.08	0.001
Nutmeg	16	0.008	1379661	1766.0	795	8.4E-09	8.37	0.140
Pimento berry (allspice)	4.5	0.13	441667	2583.8	1163	1.2E-08	12.25	0.204
Tarragon	2.1	0.008	66142	11.1	5	5.3E-11	0.05	0.0009
Banana	1	0.00001	58,000,000	5.8	3	2.8E-11	0.03	0.0005
Total food				6357	2860		30.1	0.50
Essential oil								
Basil, oil		2.6	2793	72.6	33	3.4E-10	0.34	0.01
Bay, leaves, oil		3.7	2217	82.0	37	3.9E-10	0.39	0.01
Bay, sweet oil		4.2	2473	103.9	47	4.9E-10	0.49	0.01
Citronella, oil		3	6798	203.9	92	9.7E-10	0.97	0.02
Clove, bud, oil		0.13	46067	59.9	27	2.8E-10	0.28	0.00
Nutmeg, oil		0.8	140559	1124.5	506	5.3E-09	5.33	0.09
Pimento berry, oil		13	1603	208.4	94	9.9E-10	0.99	0.02
Pimento, leaf, oil		2	7298	146.0	66	6.9E-10	0.69	0.01
Total essential oil					900.5		9.49	0.16
Added flavouring substance								
Methyl eugenol		0.99	1366	1352.3	609	6.4E-09	6.41	0.11
Sum natural flavouring complexes								0.77
Total exposure derived from added methyl eugenol (%)								14.00

[a] CIVO–TNO (1999), Duke (1992), Leung and Foster (1996), Farrell (1985), Lucas (1999).
[b] Lawrence and Shu (1993), Sheen et al. (1991), Tsai and Sheen (1987), Bobin et al. (1991), Lawrence (1994).
[c] Lucas et al. (1999).

Table 2
Oral human intake of estragole from food[a], essential oils[b] and flavour substances[c]

Food	% Volatile oil in plants	Mean concn of E in oil	1999 Reported annual volume (lb)	Intake (lb/year)	Intake (kg/year)	Intake (kg/p/d)	Intake (μg/p/d)	Intake (μg/kg bw/d)
Basil, sweet	0.5	0.27	3610861	4874.7	2194	2.3E-08	23.11	0.385
Fennel, common	6	0.03	1184856	2132.7	960	1.0E-08	10.11	0.169
Anise	4	0.035	518000	725.2	326	3.4E-09	3.44	0.057
Tarragon	0.015	0.75	66142	7.4	3	3.5E-11	0.04	0.001
Oregano	4.6	0.001	4175780	192.1	86	9.1E-10	0.91	0.015
Fennel, sweet	6	0.028	2600	4.4	2	2.1E-11	0.02	0.000
Total food				7936	3571		37.6	0.63
Essential oil								
Basil, oil		0.27	2793	754.1	339	3.6E-09	3.58	0.060
Anise, Star oil		0.037	12073	446.7	201	2.1E-09	2.12	0.035
Anise oil		0.035	13344	467.0	210	2.2E-09	2.21	0.037
Tarragon oil		0.75	435	326.3	147	1.5E-09	1.55	0.03
Fennel, sweet		0.028	2600	72.8	33	3.5E-10	0.35	0.01
Cinnamon leaf oil		0.0038	366600	1393.1	627	6.6E-09	6.61	0.11
Total essential oil					1557		16.41	0.27
Added flavouring substance								
Estragole		0.99	1234	1221.7	550	5.8E-09	5.79	0.10
Sum natural flavouring complexes								0.90
Total exposure derived from added estragole (%)								10.00

[a] CIVO–TNO (1999), Duke (1992), Leung and Foster (1996), Farrell (1985), Lucas (1999).
[b] Lawrence and Shu (1993), Sheen et al. (1991), Tsai and Sheen (1987), Bobin et al. (1991), Lawrence (1994).
[c] Lucas et al. (1999).

in the mouse in vivo in the micronucleus test (NTP, 2000).

Unscheduled DNA synthesis (UDS) in hepatocytes observed following treatment of rodents with *p*-alkoxyallylbenzene derivatives is most likely produced by cytochrome P-450-mediated metabolism of the compounds to 1′-hydroxy metabolites. The dose–response for unscheduled DNA synthesis is non-linear and, therefore, it is important to consider the dose–response relationship for formation of the 1′-hydroxy metabolite.

A marked increase in UDS was reported when estragole concentrations of 10^{-3}–10^{-5} M were incubated with primary rat hepatocytes. In an in vivo study, hepatocytes isolated 4 or 12 h after rats received a 500, 1000 or 2000 mg/kg bw dose of estragole were evaluated for unscheduled DNA synthesis. Only at 2000 mg/kg bw dose were the net grain counts greater than 5 (Muller et al., 1994).

Freshly prepared hepatocytes from F344 male rats were incubated with concentrations of methyl eugenol and estragole in the range from 10^{-6} to 10^{-2} M (Chan and Caldwell, 1992). A significant increase in UDS, as much as 2.7 times control values, occurred at concentrations in the range from 10^{-4} to 10^{-2} M for both substrates. Cytotoxicity, as measured by leakage of cytosolic lactate dehydrogenase (LDH) from hepatocytes was observed at concentrations in the range from 10^{-4} to 10^{-2} M. Incubation of the 1′-hydroxy metabolites of methyl eugenol and estragole showed increased UDS at concentrations $>10^{-5}$ M and $>10^{-5}$ to 10^{-6} M, respectively. LDH leakage occurred at 10^{-4} M for 1′-hydroxymethyl eugenol and $>10^{-4}$–10^{-5} M for 1′-hydroxy estragole. The UDS activity and cytotoxicity of the parent substances occurred at concentrations approximately an order of magnitude greater than those for their corresponding metabolites. Additionally, cytotoxicity was observed at slightly higher concentrations than those needed to induce UDS, although the differences were minimal. A clear non-linear relationship and threshold were established between dose for both substances and their metabolites and UDS activity. Similar results were obtained for methyl eugenol and estragole in an earlier study (Howes et al., 1990).

Given that estragole and methyl eugenol have been shown to form DNA adducts when laboratory rodents are exposed to high dose levels, it is not surprising that both substances and their 1′-hydroxy metabolites induce unscheduled DNA synthesis. In these studies, concentrations at which UDS occurs coincide with hepatocellular cytotoxicity.

3. Estimates of exposure from presence in food and as added flavouring substances

3.1. Presence in food

Although allylalkoxybenzene derivatives such as methyl eugenol and estragole occur at low levels in

oranges, bananas and grapefruit juice (CIVO-TNO, 1999), exposure from food occurs predominantly from the use of spices including basil, tarragon, nutmeg, mace and allspice. The concentrations of estragole or methyl eugenol in a spice show significant variation that in large part is a function of plant maturity at harvest, harvesting techniques, storage conditions, processing (e.g. drying), and method of measurement (e.g. extraction with CO_2 vs CH_2Cl_2).

Consider the influence of geographical origin on the presence of methyl eugenol in sweet and exotic (i.e. Reunion-type as found in the Comoro Islands) basil. The methyl eugenol content of the volatile oil from commercial samples of sweet and exotic basil is 0–0.5% and 1.3–2.0%, respectively (Lawrence and Shu, 1993). Other factors affecting methyl eugenol content include distribution within the plant, plant maturity, harvest time and drying conditions. The methyl eugenol content for the same cultivar of sweet basil was in the range of 0.6–2.4% for the leaf, flower and stem with highest concentrations found in the leaf (Tsai and Sheen, 1987; Sheen et al., 1991). Methyl eugenol content of sweet basil leaves of the Genovese Gigante variety are inversely proportional to plant height (maturity). Methyl eugenol content in the essential oil of sweet basil decreased from essentially 100–10% as the plant matured from 3–6 cm to 12–6 cm (Miele et al., 2001). Therefore, early harvested basil provides significantly increased exposure to methyl eugenol.

For basil harvested at the optimum time and stored over various periods, the concentration of methyl eugenol in fresh leaves was 0.05%, in leaves stored 2 months it was 20.4%, and in leaves crushed and frozen at 20 °C it was 1.6% (Bobin et al., 1991).

Although the above factors make a significant impact on methyl eugenol intake from consumption of basil, data on a wide range of commercially available oils can be used to determine an average daily intake. In more than 200 analyses of sweet basil oils produced from plants in North America, the volatile oil content in 102 samples was 0.01–0.30% (Lawrence et al., 1994) and the methyl eugenol content of the oil was 1.04–2.66% (Lawrence et al., 1988). Similarly, in allspice (i.e. pimento berry), the volatile oil content was 4.0–4.5% and methyl eugenol content of the oil was 2.9–13.0% (Green and Espinosa, 1988; Lawrence and Shu, 1993).

From a conservative viewpoint, average intake of methyl eugenol from consumption of spices may then be determined from the methyl eugenol content of the volatile oil obtained from the various spices. For instance, allspice may contain up to 4.5% oil with methyl eugenol content of 13%. Since the annual volume of allspice consumed is 200,500 kg/yr in the US (Lucas et al., 1999), the annual amount of methyl eugenol consumed as a result of eating allspice flavoured food would be approximately 1170 kg ($0.045 \times 0.13 \times 200{,}500$) (see Table 1). The average daily intake of methyl eugenol from consumption of allspice is estimated to be 0.2 μg/kg bw per day (1170 kg/yr $\times 1/264 \times 10^6 \times 1/60$ kg $\times$ 1/365 days $\times 10^9$ μg/kg). Estimates of the total average daily intake of methyl eugenol and estragole from consumption of traditional foods can be found in Tables 1 and 2, respectively. The total average daily intakes of methyl eugenol and estragole from consumption of spices are estimated to be 0.5 and 0.63 μg/kg bw/day, respectively. Assuming that only 10% of the population consumed all of the food containing methyl eugenol and estragole (i.e. "eaters only"), daily per capita intake would be 5.0 and 6.3 μg/kg bw.

3.2. Exposure from addition of essential oils and added as a flavouring substance

Methyl eugenol and estragole are also added to food either directly as flavouring substances or as constituents of added essential oils. They have a taste described as spice, cinnamon and clove, mouth tingle, fresh, peppery and woody. Methyl eugenol and estragole are used for the modification of spice flavours and seasonings for condiments and meats as well as in heavy fruit, root beer and anise-type flavours. Based on the conservative (i.e. high) estimates of the methyl eugenol and estragole content of the various essential oils, and the annual volume of use of these oils (NAS, 1987, 1981, 1975, 1970; Lucas et al., 1999), the average daily intake of methyl eugenol and estragole from use of essential oils can be estimated (see Tables 1 and 2, respectively). An estimate of the intake of methyl eugenol intentionally added as a flavouring substance is based on its reported annual volume of use (NAS, 1987, 1981, 1975, 1970; Lucas et al., 1999).

As indicated in Tables 1 and 2, consumption of spices and essential oils derived from spices is responsible for the majority of intake of methyl eugenol and estragole. Basil, nutmeg, allspice, tarragon, anise and bitter fennel are major sources of exposure. Mean daily per capita intake of methyl eugenol from all sources is approximately 0.8 μg/kg bw per day with more than 85% derived from consumption of basil, allspice, nutmeg and their essential oils. Mean daily per capita intake of estragole from all sources is approximately 1.0 μg/kg bw per day with approximately 90% derived from consumption of basil, fennel, tarragon, anise and their essential oils. Based on the conservative assumption that only 10% of the US population consumed foods containing methyl eugenol or estragole, the estimated daily per capita intake ("eaters only") of either substance is estimated to be less than 10 μg/kg bw per day.

3.3. Special eaters of foods containing methyl eugenol

There are specialised eating groups of methyl eugenol. Pesto eaters are exposed to some of the highest levels of

methyl eugenol by far, because fresh pesto is prepared from a large quantity of fresh sweet basil. Considering that a single portion of pesto may contain up to 10 g of basil (oil content 0.5%) and the Genovese Gigante cultivar of basil, the most commonly used in pesto preparation in north-western Italy, contains greater than 40% methyl eugenol, a typical serving of pesto may contain 250 μg/kg of methyl eugenol (Miele et al., 2001). Therefore, although normal exposure to methyl eugenol may be of the order of 1–10 μg/kg bw/day, exposures at least 10 times higher may be experienced by specialised eating groups.

4. Safety assessment for exposure to estragole and methyl eugenol from use in food and as intentionally added flavouring substances

4.1. Introduction

Beginning with the seminal research on allylalkoxylbenzene derivatives (Long and Jenner, 1963; Hagan et al., 1967; Borchert et al., 1973; Miller et al., 1979, 1983), there has been ongoing interest in the carcinogenic potential of these naturally occurring substances and the mechanism by which they induce carcinogenicity in animals. Methyl eugenol, estragole, and for that matter safrole have been and will continue to be consumed as a normal part of a traditional diet. They occur at highest levels in spices, which are consumed at low levels in food. They are also present in a limited number of foods at very low levels (CIVO–TNO, 1999). Recent data (Barr et al., 2000) indicating that methyl eugenol is essentially ubiquitous in humans, acknowledge the fact that humans are regularly exposed to methyl eugenol in the diet and, in all probability, via other routes of exposure. Although the preponderance of data in rodents indicates that, at relatively high levels of exposure, methyl eugenol, estragole and other structurally related substances exhibit a carcinogenic potential in rodents, a growing database of information also indicates that, at low levels of exposure occurring through consumption of a traditional diet, these substances present no significant risk to humans. Current scientific evidence strongly supports a non-linear relationship between dose and toxicity of allylalkoxybenzene derivatives.

4.2. Interpretation of pharmacokinetic and metabolic data

Comparison of peak plasma levels achieved in rodents chronically exposed to high dose levels in the NTP study with levels detected in the background population (Barr et al., 2000) provides a basis for interpreting the difference in exposure between laboratory rodents used in bioassays and humans. Rats given the lowest dose of methyl eugenol (37 mg/kg bw per day) in the NTP study exhibited peak plasma levels (1–10 μg/ml), greater than 10,000 times the mean serum levels of methyl eugenol detected in humans in the NHANES survey (0.000025–0.00010 μg/ml) (NTP, 2000) or the ginger snap study (S. Masten, personal communication, 2000).

Metabolic activation undoubtedly played an important role at these high dose levels in rodent studies. Dose-dependent pharmacokinetics indicate that at low dose levels estragole and methyl eugenol are rapidly cleared from the system, with *O*-demethylation being the major metabolic route. Metabolic shifting (*O*-demethylation to 1′-hydroxylation) results in increased formation of the hepatotoxic 1′-hydroxy metabolite at higher dose levels. Quantitatively, the amount of 1′-hydroxy metabolite excreted by rats given 1000 mg estragole/kg bw dose level is approximately 160,000 times the level excreted at 0.05 mg estragole/kg bw in rats (Zangouras et al., 1981). This is a function of a 20,000-fold increase in dose and an approximately six- to eight-fold increase in metabolic activation. Based on the reasonable assumption that methyl eugenol and estragole exhibit parallel dose-dependent metabolism and the pattern of urinary excretion of the 1′-hydroxy metabolite, the level (2 mg/kg bw) of 1′-hydroxy metabolite formed and excreted at the lowest dose level of methyl eugenol (37 mg/kg bw/day) in the NTP 2-year bioassay is at least 50,000 times the level (0.00003 mg/kg bw per day) produced in humans exposed to methyl eugenol from consumption of food.

The lifetime body burden of methyl eugenol in rats in the low dose in the NTP study (37.5 mg/kg bw per day for 728 days) is estimated to be 27.3 g/kg, while that in humans is at most only 0.219 g/kg (0.010 mg/kg bw per day for 365 days over 60 years). Based principally on the difference in levels of exposure and, to a smaller extent, metabolic switching, human exposure to methyl eugenol and the proximate hepatotoxin, 1′-hydroxy methyl eugenol, is at least three orders of magnitude less than exposure of laboratory animals exhibiting toxicity.

4.3. Interpretation of enzyme induction, protein adduct, and DNA adduct studies

Based on research performed during the late 1990s (Gardner et al., 1997), significant induction of the CYP-450 activation pathway is anticipated in rats in the NTP bioassay (NTP, 2000) at dose levels of 37, 75 and 150 mg/kg bw/day. The induction of CYP-450 isoenzyme 2E1 is linked sequentially to increased production of the 1′-hydroxy metabolite, cellular protein adduct formation, effects on cell growth, hepatotoxicity, DNA adduct formation, and eventually carcinogenicity. It appears that at dose levels below 1–10 mg/kg bw, rodents and humans metabolise estragole or methyl eugenol (i.e. *p*-methoxyallylbenzene derivatives) primarily by *O*-demethylation and side-chain oxidation to yield metabolites

that are effectively excreted primarily in the urine. As dose levels are increased, *O*-demethylation decreases and induction of CYP-450 (CYP2E1 and probably CYP-2C6) catalysed 1′-hydroxylation occurs. CYP-450 induction of the 1′-hydroxylation pathway by methyl eugenol itself does not occur in rats given 10 mg/kg bw or less orally for 5 days but does occur at higher dose levels (30–300 mg/kg bw/day). At these higher dose levels, increased hepatocellular concentrations of sulfate ester of the 1′-hydroxy metabolite dissociate into a reactive electrophilic species that covalently binds microsomal protein and DNA.

A microsomal protein adduct (44 kDa) forms at low doses (10 and 30 mg/kg bw/day) and an assortment of protein adducts form at higher doses (100 and 300 mg/kg bw/day). DNA adducts of methyl eugenol and estragole have been detected at dose levels slightly above 10 mg/kg bw. Seminal research (Miller et al., 1983; Phillips et al., 1984; Randerath et al., 1984; Wiseman et al., 1987) relating the extent of DNA adduct formation to the incidence of hepatomas in various strains of mice provides evidence that (1) there is no direct relationship between DNA adduct formation and rates of tumourigenicity; and (2) DNA adduct repair of these types of covalently bound adducts operates in vivo, as indicated by the rapid decrease in adduct formation following exposure to methyl eugenol or estragole. No studies have been reported on the levels of DNA adducts in target or non-target tissues for methyl eugenol, estragole or safrole at typical levels of human exposure.

It appears that at dose levels below 10 mg/kg bw/day, the extent of 1′-hydroxylation is decreased (Zangouras et al., 1981; Sangster et al., 1987). At higher dose levels, induction of activation enzymes (2E1) and hepatocellular protein adduct formation become significant (Gardner et al., 1997). Presumably, the reactive species are effectively trapped by microsomal proteins, glutathione and other reactants, probably at or near the site of formation. However, chemical characterisation of this reaction and information on the concentration at which these biochemical events initiate hepatotoxicity are as yet unknown. Additional dose–response and metabolism data at dose levels (< 10 mg/kg bw) would provide key data for a physiologically-based pharmacokinetic model. Such a model would yield a quantitative understanding of the internal dosimetry of methyl eugenol and estragole and their 1′-hydroxy metabolites in rodents and humans.

4.4. Interpretation of the 2-year bioassay with methyl eugenol

The intention of the NTP 2-year bioassay is to identify the carcinogenic hazard regardless of the dose levels or route of exposure. In no way is it intended to be the sole basis for a risk assessment without additional perspective provided by studies on the relevance at human exposure levels. Even viewed from this perspective, the methyl eugenol bioassay was compromised by inappropriately high dose levels, administered by gavage, that cause significant hepatotoxicity, gastric damage, and malnutrition in both mice and rats. The presence of *H. hepaticus* in the livers of mice was also thought to have confounded the interpretation of the findings. Hepatic tumours occurred in severely damaged livers while the neuroendocrine tumours were likely to have resulted from endocrine responses to chronic gastric damage.

At dose levels of methyl eugenol at which hepatic tumours occurred in rats, non-neoplastic liver changes such as liver and hepatocyte enlargement, necrosis, chronic inflammation, periportal fibrosis and nodular or adenomatoid hyperplasia, were invariably present. Such recurrent liver damage, in particular chronic inflammation and hyperplasia undoubtedly altered methyl eugenol metabolism and may have strongly enhanced the likelihood of DNA damage, fixation of relevant DNA damage and progression of initiated/preneoplastic cells to cancer. Therefore, the hepatotoxicity induced by high dose levels of methyl eugenol most probably plays a very significant, if not an essential, role in the formation of hepatic tumours. If in humans, exposure to high levels of methyl eugenol were to be accompanied by recurrent liver tissue damage and hyperplasia, methyl eugenol might possibly induce liver cancer in humans. However, if dose levels of methyl eugenol in humans are less than those needed to induce hepatotoxicity (most probably somewhere in the range of 1–10 mg/kg bw/day), exposure of humans to such non-hepatotoxic levels can be assumed to be associated with a very low, probably zero, cancer risk. The absence of carcinogenicity and the lack of significant liver toxicity at low dose (25 and 5 mg/kg bw) in a 2-year dietary study with safrole (Long et al., 1963) provides evidence that, in all likelihood, a rodent no-observed-adverse-effect level (NOAEL) can be established for methyl eugenol and estragole in the range of 1–10 mg/kg bw per day. Since human exposure to methyl eugenol from consumption of food (0.01 mg/kg bw/day) is at least two orders of magnitude lower than the no-observed-hepatotoxic-effect level in rodents, in terms of cancer risk, such a methyl eugenol exposure in humans is considered to be negligibly small.

Similar to the liver, neoplastic effects in the glandular stomach must be evaluated in the context of severe gastric damage. Toxicokinetic studies revealed rapid absorption of methyl eugenol rats and mice with peak plasma levels obtained within the first 5 min (NTP, 2000. The bioavailability of methyl eugenol administered orally increased in a non-linear fashion with dose, suggesting saturable first-pass metabolism or altered absorption and metabolism due to gastric and hepatic damage. Introduction of a bolus of test material into the

glandular stomach exposes the stomach and upper intestines to high concentrations of methyl eugenol and leads to higher peak blood plasma levels, and increased metabolic demand than slower steady absorption of substance from the feed. The results of NTP 2-year bioassays and related toxicokinetic studies (Yuan et al., 1995) in which the test material was administered by gavage and in the feed (NTP, 1986, 1993) have clearly demonstrated that peak plasma levels are significantly higher in gavage-dosed animals.

It has been reported (Poynter and Selway, 1991; Thake et al., 1995) that chronic gastrin stimulation of the enterochromaffin-like cells results in the formation of neuroendocrine tumours of the glandular stomach. Parietal cell cytotoxicity and atrophy of the fundic mucosa, hypochlorhydria and hypergastrinemia typically accompany the tumourigenic response. In the case of methyl eugenol, it is likely that severe damage to the glandular stomach was the result of hypo- or achlorhydria leading to chronic gastrinemia, a proliferative and ultimately carcinogenic stimulus to the gastric neuroendocrine cells in the NTP bioassay. Chronic toxicity to the glandular stomach was very likely related to anorexia and abnormal digestion and absorption leading to malnutrition and further organ damage and dysfunction. Gastric damage almost certainly significantly affected the absorption, metabolism and excretion of methyl eugenol, leading to hepatic, renal and possibly other organ damage. It is recommended that any re-evaluation of the toxicity to the glandular stomach consider administration of the test substance micro-encapsulated in the feed.

4.5. Conclusions

High doses of methyl eugenol are carcinogenic in rodents. This has been observed in several different studies in mice and rats, newborns and adults. Repetitive ip administration for 20 days of high concentrations of safrole, estragole or methyl eugenol to preweanling or weanling mice induced liver tumours at approximately 10–12 months (Borchert et al., 1973; Miller et al., 1979, 1983; Wiseman et al., 1987). Preweanling animals were more sensitive to tumourigenesis. Similar effects are seen at higher dose levels with methyl eugenol and safrole administered either by gavage or in the diet (Long and Jenner, 1963; Hagan et al., 1967; NTP, 2000). In these studies, evidence of carcinogenicity was concurrent with evidence of chronic hepatotoxicity. The lowest dose of methyl eugenol administered by gavage at which carcinogenicity and hepatotoxicity was reported in rodents in the NTP 2-year bioassay was 37 mg/kg/day. In a separate 2-year dietary study, safrole was not carcinogenic when administered in the diet at 25 or 5 mg/kg bw/day, although mild hepatotoxicity was reported even at these low doses (Long and Jenner, 1963). Therefore, no definitive study has as yet been performed in the absence of hepatotoxicity using the oral route of exposure.

Based on the results of these studies, dose-dependent hepatotoxicity induced by methyl eugenol, safrole and other allylalkoxybenzene derivatives is associated with the formation of hepatic tumours and is likely to be a necessary step in carcinogenesis. Daily intake of doses of methyl eugenol that are carcinogenic in rodents following chronic gavage administration are approximately 1000-fold higher than the typical dietary intake of methyl eugenol. Since the amount of methyl eugenol added as a flavouring constituent accounts for approximately 10% of dietary intake, its potential to induce hepatotoxicity is expected to be negligible, possibly zero. As with all substances administered at high dose in carcinogenesis assays, there is uncertainty about the shape of the dose–response curve at doses that are typical of normal human exposure. This uncertainty is compounded in the case of methyl eugenol by the fact that it was administered chronically in the NTP bioassay by gavage, which clearly induced gastric toxicity.

Methyl eugenol has been detected in the plasma of humans, indicating that methyl eugenol and other structurally related substances are absorbed from the diet and distributed. Methyl eugenol is rapidly metabolised by *O*-demethylation, epoxidation and 1′-hydroxylation with the 1′-hydroxy metabolite being the proximate hepatotoxic and carcinogenic agent. The daily production of the 1′-hydroxy metabolite by rodents at high dose levels in chronic studies is orders of magnitude greater than those formed in humans at typical dietary intake. Less than 0.3% of a typical dietary dose of estragole is metabolised and excreted in the urine of humans as the 1′-hydroxy metabolite while as much as 40% of carcinogenic doses of safrole can be accounted for in the urine of mice. The increase in 1′-hydroxylation has been related to dose-dependent induction of selected CYP-450 isoenzymes (2E1) (Sharma et al., 2001).

Following metabolism, methyl eugenol forms adducts to DNA and protein. Dose-dependent protein adducts have been isolated from rats receiving repeated doses of methyl eugenol. These have not been chemically characterised. The principal DNA adduct originates from the 1′-hydroxy metabolite through coupling of an allylic carbocation to the exocyclic amino group (N^2) of deoxyguanosine residues. DNA adducts have been detected in the livers of mice at doses that induce tumour formation. Comparison of adduct levels induced in preweanling and weanling mice and the kinetics of their disappearance indicates that these adducts are more slowly removed from preweanling animals. This may explain the increased sensitivity of newborns to the carcinogenic effects of high dose levels of methyl eugenol.

Curiously, methyl eugenol is not strongly mutagenic in bacterial or yeast test systems with metabolic activation. 1′-Acetoxymethyl eugenol, a chemical model for the ultimate activation metabolite, 1′-sulfo-oxymethyl eugenol, is mutagenic in *Salmonella* although a non-linear dose–response was reported. The mutagenic potencies of the N^2-deoxyguanosine adducts of methyl eugenol have not been directly tested in site-specific mutagenesis assays. Structurally analogous DNA adducts formed by reaction of the epoxide of styrene oxide with the exocyclic amino group of deoxyadenosine are weakly mutagenic in site-specific assays (Latham et al., 1993). Their activities in comparable assays are approximately 10–100-fold lower than that of strongly mutagenic DNA adducts such as O^6-methyldeoxyguanosine. It would be highly desirable to quantify, in parallel, the levels of methyl eugenol-DNA adducts in the liver as a surrogate for genotoxicity.

Both the qualitative and quantitative aspects of the molecular disposition of methyl eugenol and estragole and their associate toxicological sequelae have been relatively well defined from mammalian studies. Several studies have clearly established that the profiles of metabolism, metabolic activation and covalent binding are dose dependent and that their relative importance diminishes markedly at low levels of exposure (i.e. these events are not linear with respect to dose). In particular, rodent studies show that these events are minimal probably in the dose range of 1–10 mg/kg bw, which is approximately 100–1000 times the anticipated human exposure to these substances. For these reasons it is concluded that present exposure to methyl eugenol and estragole resulting from consumption of food, mainly spices and added as such, does not pose a significant cancer risk. Nevertheless, in the interim further studies are needed to confirm both the nature and implications of the dose–response curve in rats at low levels of exposure to methyl eugenol and estragole.

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