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Review Section

The FEMA GRAS Assessment of *trans*-Anethole Used as a Flavouring Substance

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Summary—This publication is the fourth in a series of safety evaluations performed by the Expert Panel of the Flavour 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 *trans*-anethole (i.e. 4-methoxypropenylbenzene) as a flavouring substance is critically evaluated by the FEMA Expert Panel. The evaluation uses a mechanism-based approach in which production of the hepatotoxic metabolite anethole epoxide (AE) is used to interpret the pathological changes observed in different species and sexes of laboratory rodents in chronic and subchronic dietary studies. Female Sprague–Dawley rats metabolize more *trans*-anethole to AE than mice or humans and, therefore, are the most conservative model for evaluating the potential for AE-induced hepatotoxicity in humans exposed to *trans*-anethole from use as a flavouring substance. At low levels of exposure, *trans*-anethole is efficiently detoxicated in rodents and humans primarily by *O*-demethylation and ω-oxidation, respectively, while epoxidation is only a minor pathway. At high dose levels in rats, particularly females, a metabolic shift occurs resulting in increased epoxidation and formation of AE. Lower activity of the "fast" acting detoxication enzyme epoxide hydrolase in the female is associated with more pronounced hepatotoxicity compared to that in the male. The continuous intake of high dose levels of *trans*-anethole (i.e. cumulative exposure) has been shown in dietary studies to induce a continuum of cytotoxicity, cell necrosis and cell proliferation. In chronic dietary studies in rats, hepatotoxicity was observed when the estimated daily hepatic production of AE exceeded 30 mg AE/kg body weight. In female rats, chronic hepatotoxicity and a low incidence of liver tumours were reported at a dietary intake of 550 mg *trans*-anethole/kg body weight/day. Under these conditions, daily hepatic production of AE exceeded 120 mg/kg body weight. Additionally, neither *trans*-anethole nor AE show any evidence of genotoxicity. Therefore, the weight of evidence supports the conclusion that hepatocarcinogenic effects in the female rat occur via a non-genotoxic mechanism and are secondary to hepatotoxicity caused by continuous exposure to high hepatocellular concentrations of AE. *trans*-Anethole was reaffirmed as GRAS (GRASr) based on (1) its low level of flavour intake (54 μg/kg body weight/day); (2) its metabolic detoxication pathway in humans at levels of exposure from use as a flavouring substance; (3) the lack of mutagenic or genotoxic potential; (4) the NOAEL of 120 mg *trans*-anethole/kg body weight/day in the female rat reported in a 2+-year study which produces a level of AE (i.e. 22 mg AE/kg body weight/day) at least 10,000 times the level (0.002 mg AE/kg body weight/day) produced from the intake of *trans*-anethole from use as a flavouring substance; and (5) the conclusion that a slight increase in the incidence of hepatocellular tumours in the high dose group (550 mg *trans*-anethole/kg body weight/day) of female rats was the only significant neoplastic finding in a 2+-year dietary study. This finding is concluded to be secondary to hepatotoxicity induced by high hepatocellular concentrations of AE generated under conditions of the study. Because *trans*-anethole undergoes efficient metabolic detoxication in humans at low levels of exposure, the neoplastic effects in rats associated with dose-dependent hepatotoxicity are not indicative

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of any significant risk to human health from the use of *trans*-anethole as a flavouring substance.

Abbreviations: ADI = acceptable daily intake; AE = anethole epoxide; 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; NAS = National Academy of Sciences; NOAEL = no-observable-adverse-effect level; NTP = National Toxicology Program; LD_{50} = median lethal dose; MLA = mouse lymphoma cells; ppm = parts per million; SAL = *Salmonella typhimurium*; SCE = sister chromatid exchanges; UDS = unscheduled DNA synthesis.

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1.0. Explanation

1.1. Introduction

trans-Anethole (FEMA No. 2086) is an alkoxypropenylbenzene derivative with important use as a flavouring substance in baked goods, candy, ice cream, chewing gum and alcoholic beverages. It is the major volatile component in sweet and bitter fennel, and anise. In 1989, it was reported that chronic intake of high dose levels of *trans*-anethole in the female rat was associated with hepatotoxicity and a low incidence of liver tumours (Truhaut *et al.*, 1989). Subsequent studies on the genotoxicity, toxicity, pharmacokinetics and metabolic fate of *trans*-anethole in laboratory animals and humans have been performed and are used here in a mechanism-based safety evaluation to interpret the observed hepatotoxicity and related tumorigenic effects. Changes reported in the liver of laboratory rodents exposed to *trans*-anethole in the diet for periods up to 833 days (i.e. 2+ years) can be quantitatively linked to a metabolic intoxication pathway of *trans*-anethole yielding AE.

1.2. Regulatory status

In 1965, the FEMA Expert Panel reviewed *trans*-anethole and concluded that it is "generally recognized as safe" (GRAS) under conditions of intended use as a flavouring substance in food (Hall and Oser, 1965). In 1979, the Panel again evaluated the available data and affirmed the GRAS status of *trans*-anethole for use as a flavouring substance in food (GRASa). In 1997, the FEMA Expert Panel performed a third comprehensive review of all scientific data relevant to the safety evaluation of *trans*-anethole from use as a flavouring substance in food. This document contains the Panel's interpretation of those data.

2.0. Biological data

2.1. Biochemical aspects

2.1.1. Absorption, distribution, biotransformation and excretion

2.1.1.1. Introduction

In mice, rats and humans, orally administered *trans*-anethole and other alkoxypropenylbenzene derivatives are rapidly absorbed from the gastrointestinal tract and completely metabolized mainly in the liver to yield polar acidic metabolites, which are conjugated and eliminated primarily in urine. Carbon dioxide also is produced, primarily by *O*-demethylation, and is eliminated in expired air (Fritsch *et al.*, 1975; Le Bourhis, 1968, 1970, 1973b; Solheim and Scheline, 1973, 1976; Strolin-Benedetti and Le Bourhis, 1972). Major urinary and intermediary metabolites formed in the rat pass into bile with subsequent entry into enterohepatic circulation (Solheim and Scheline, 1976).

Pharmacokinetic and metabolic information for *trans*-anethole and related substances indicate that alkoxypropenylbenzene derivatives undergo detoxication in mammals by three principal pathways (see Fig. 1):

1. ω-Oxidation of the propenyl side-chain to yield a cinnamyl alcohol derivative which is successively oxidized to the corresponding acid. The acid may be conjugated with glycine and be excreted or undergo β-oxidation, cleavage and conjugation to the corresponding hippuric acid derivative.
2. *O*-Demethylation of the *p*-methoxy substituent of *trans*-anethole or one of its ω-oxidation metabolites to yield the corresponding phenolic derivative which may be excreted as the sulfate or glucuronic acid conjugate.
3. Epoxidation of the side-chain double bond to yield AE which either may be hydrolysed by cytosolic epoxide hydrolase to yield the corresponding diol or conjugated with glutathione (GSH) and excreted. *Cis* and *trans* AE are thermally stable epoxides which have been synthesized from *cis*- and *trans*-anethole, respectively, via two synthetic routes (Mohan and Whalen, 1993). AE is commercially available (Glidco, Inc.). AE is converted chemically to the corresponding diol under mildly acidic aqueous conditions (Mohan and Whalen, 1993; Schulz and Treibs, 1944) which, along with the glutathione conjugate, have been isolated as urinary metabolites of *trans*-anethole in laboratory rodents and humans. Similarly, other alkenylbenzene derivatives (e.g. β-methylstyrene and isoeugenol) have been reported to form stable epoxide derivatives upon treatment with peracids (Foltz and Witkop, 1957).

The three pathways identified for *trans*-anethole have been characterized with other alkoxypropenylbenzene derivatives and intermediary metabolites such as *p*-methoxycinnamic acid (a metabolite of *trans*-anethole) (Solheim and Scheline, 1973) and 3,4-dimethoxycinnamic acid (a metabolite of methyl isoeugenol) (Solheim and Scheline, 1976). However, only in the case of *trans*-anethole has the influence of species, sex, dose, and duration on metabolic pathway been thoroughly evaluated.

2.1.1.2. Humans

In two male volunteers, 81% of a single dose of 1 mg *trans*-[methoxy-^{14}C]anethole was either excreted in the urine or exhaled as CO_2 within 8 hours. After 48 hours, total recovery of radioactivity reached 88%. Within the first 8 hours, 21% of the dose was metabolized by *O*-demethylation and excreted as $^{14}CO_2$ in exhaled air. Within 24 hours, 60% of the dose was excreted in the urine as ω-oxidation metabolites, primarily *p*-methoxybenzoic acid (3.5%) and its glycine conjugate *p*-methoxyhippuric acid (56%). Approximately 3% of the

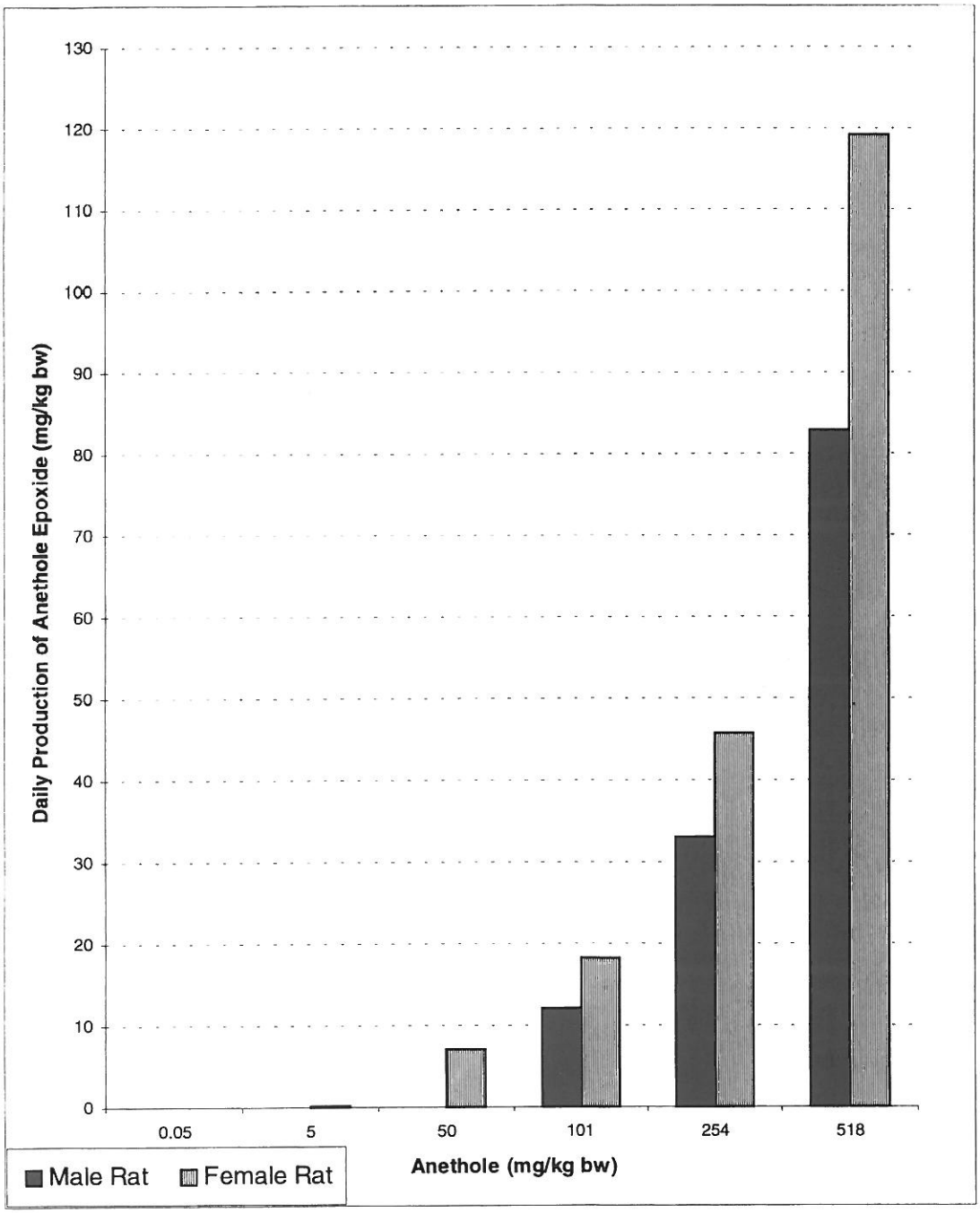

Fig. 1. Daily anethole epoxide production in male and female rats.

dose was metabolized to an epoxide, which was excreted as a mixture of diastereomeric diols of 1-(4′-methoxyphenyl)propane-1,2-diol and as the mercapturic acid conjugate (< 0.2%) (Sangster *et al.*, 1987). This is the only study in which AE metabolites were isolated and identified. In other studies at higher dose levels, only ω-oxidation and *O*-demethylation metabolites were quantified.

Five volunteers with body weights of 62–77 kg received oral doses of 1, 50 or 250 mg *trans*[methoxy-^{14}C]anethole (approx. 0.015, 0.87 or 4 mg/kg body weight) separately on three occasions. The bulk of the radioactivity was eliminated in expired air and urine within the first 8 hours. Over the dose range studied, dose had no effect on the rate or route of excretion. Greater than 90% of

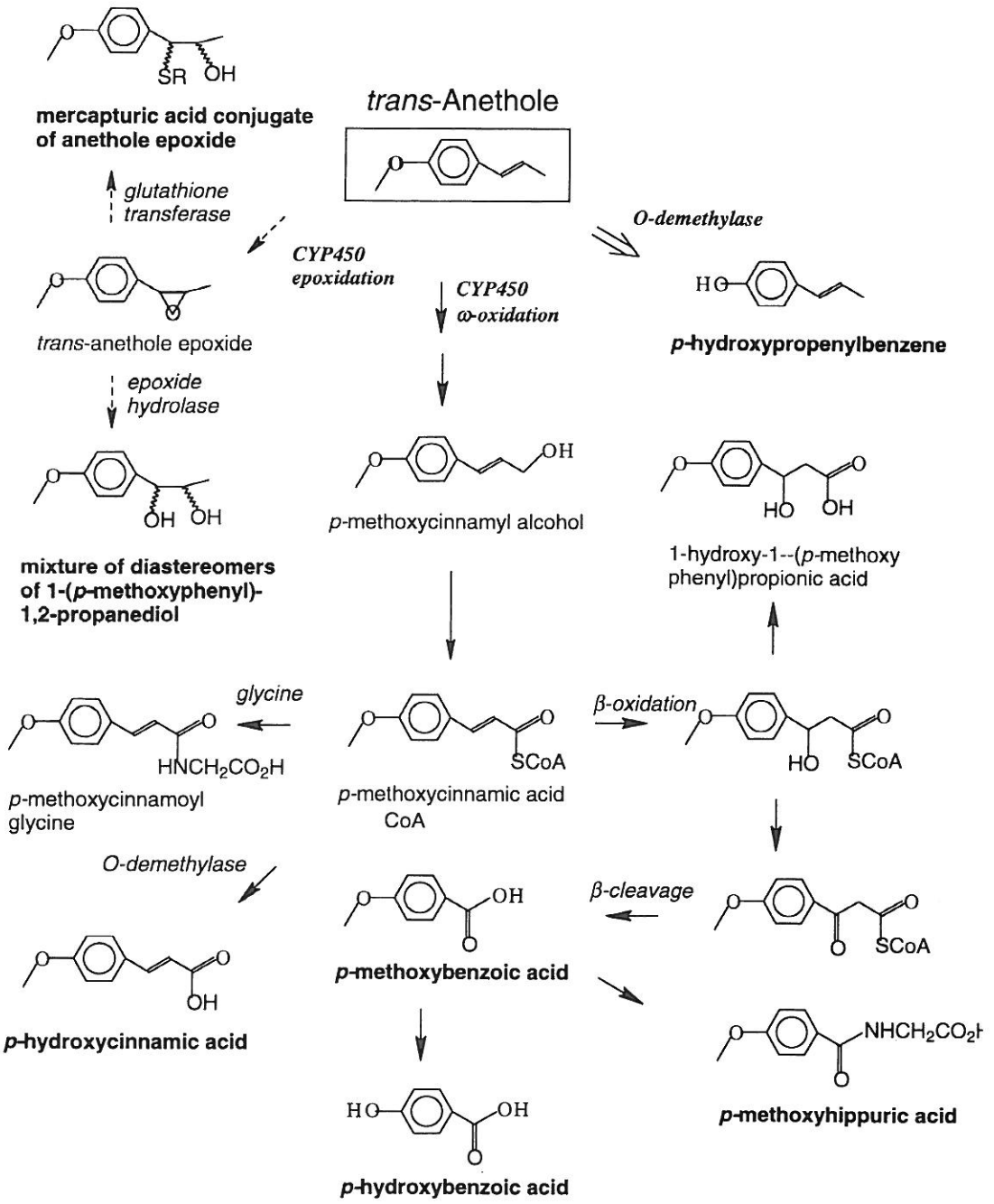

Fig. 2. Metabolism of *trans*-anethole in humans, rats and mice.

urinary metabolites (54–69% of the dose) was comprised of the glycine conjugate of 4-methoxyhippuric acid. Based on the level of $^{14}CO_2$ in exhaled air, *O*-demethylation accounted for 13–17% of the dose. However, the authors noted that the method of CO_2 collection led to an underestimate of excretion via the exhalation route and was probably responsible for the incomplete total recovery (68–86%) of administered radioactivity (Caldwell and Sutton, 1988).

In a third study, five volunteers were given a single 500 mg dose (approx. 12 mg/kg body weight) of *trans*-anethole. Metabolism occurred primarily by ω-oxidation, β-oxidation, and cleavage to yield *p*-methoxybenzoic acid (52% of the total dose) and *p*-hydroxybenzoic acid (5% of the total dose), which were excreted in the urine within 24 hours (Le Bourhis, 1973b). The data in these three studies demonstrate that at dose levels ranging from 0.05 to 12 mg/kg body weight/day, *trans*-anethole under-

goes metabolic detoxication by ω-oxidation and *O*-demethylation pathways.

2.1.1.3. Mice

In mice, *trans*-anethole is rapidly absorbed, metabolized and excreted primarily in the urine. Less than 50% of a 200 mg/kg body weight dose of *trans*-[^{14}C]anethole* given to five CD-1 mice was detected in the blood after 1 hour and less than 10% was detected after 5 hours (Le Bourhis, 1973b). 3 hours after mice were given a 100 mg/kg body weight oral dose of *trans*-[^{14}C]anethole†, radioaccumulation occurred mainly in the liver, intestine, kidney and bile. After 24 hours, the highest levels of radioactivity occurred in the intestine, bladder and bile (Strolin-Benedetti and Le Bourhis, 1972).

Four male CD-1 mice were given a single ip injection of 50 mg *trans*-[methoxy-^{14}C]anethole/kg body weight (Sangster *et al.*, 1984a). More than 85% of the radiolabel was recovered over 24 hours in the urine (37%), in the air as expired $^{14}CO_2$ (47%), and in the faeces (< 2%). *O*-Demethylation to yield $^{14}CO_2$ accounted for 47% of the dose. 10 urinary metabolites were formed from ω-oxidation, *O*-demethylation and epoxidation. Metabolites derived from ω-oxidation of the side-chain included mainly *p*-methoxyhippuric acid (≈17.8%). Metabolites derived from epoxidation of the alkene (≈6.2%) included glucuronic acid conjugates of a diasteriomeric mixture of diols (2 isomers of 1-(4′-methoxyphenyl)propane-1,2-diol), 1-(4′-methoxyphenyl)-1-oxopropan-2-ol, and a GSH conjugate (*S*-(1-(4′-methoxyphenyl)-2-hydroxypropane)-*N*-acetylcysteine). In addition to these ^{14}C-labelled metabolites, *O*-demethylated metabolites, such as *p*-hydroxypropenylbenzene, were also excreted as the glucuronic acid conjugate in the urine (Fig. 2).

The effect of dose on the metabolism of *trans*-anethole was studied in CD-1 mice (four/group) given a single dose of 0.05, 5, 50 or 1500 mg *trans*-[methoxy-^{14}C]anethole/kg body weight by ip injection. Approximately 85% of the dose was recovered at all dose levels. The increase in dose caused a significant shift in metabolism from *O*-demethylation metabolites (71.8% at 0.05 mg/kg body weight to 34.6% at 1500 mg/kg body weight) to ω-oxidation metabolites (10.4% at 0.05 mg/kg body weight to 43.8% at 1500 mg/kg body weight) and to a lesser extent, epoxidation metabolites (1.6% at 0.05 mg/kg body weight to 8.8% at 1500 mg/kg body weight) (see Table 1). Additionally, complete elimination of radioactivity was slower at higher dose levels, requiring 72 hours at 1500 mg/kg body weight *v.* 24 hours at lower doses (Sangster *et al.*, 1984b).

In a related study on the influence of dose, sex and pre-feeding on the metabolism of *trans*-anethole, groups of six male and female CD-1 mice were maintained for 21 days on diets containing 0, 0.05, 0.1, 0.25 or 0.5% *trans*-anethole resulting in an average daily intake of 0, 62, 140, 296 or 426 mg/kg body weight, respectively. A control group was maintained for each of the four groups of mice that received *trans*-anethole for 21 days. All groups of control and pre-fed mice were then given a single oral dose of *trans*-[^{14}C]anethole by gavage at a dose equivalent to the pre-feeding level determined from week 3 of the study.

Within 24 hours, all control and pre-fed groups of mice excreted the majority (75–90%) of radioactivity in the urine. No discernible dose-dependent shift in excretion pathway occured in either control or pre-fed mice at daily dose levels between 62 and 426 mg/kg body weight. The proportions of urinary metabolites derived from *O*-demethylation (mainly *p*-hydroxypropenylbenzene), side-chain ω-oxidation (mainly *p*-methoxyhippuric acid) and epoxidation (anethole diols and GSH conjugate) remained relatively constant over the dose range in both control and pre-fed groups (Fig. 2) indicating that pre-feeding with *trans*-anethole does not alter the capacity of mice to metabolize and eliminate *trans*-anethole.

At the highest dose, groups of pre-fed males excreted more ω-oxidation metabolites than females. Glycine conjugation of *p*-methoxycinnamic acid decreased with increasing dose and pre-feeding, which suggests that glycine conjugation is capacity limited at higher dose levels. The effect was more pronounced in females, implying that the female has a lower capacity for glycine conjugation. The decrease in glycine conjugation was accompanied by a dose-related increase in the formation of GSH conjugates. In control and pre-fed females at the high dose, an increase (2–6%) in epoxidation metabolites was observed (mainly as GSH conjugates) (Bounds, 1994; Bounds and Caldwell, 1992, 1996).

No significant dose-dependent switch to an epoxidation pathway was evident in repeated dose studies in the mouse at dose levels up to 1500 mg/kg body weight/day. Only a slight (but significant) increase in epoxide conjugates was observed at the highest dose, most notably in the female. Clearly, the major metabolic routes in mice consist of ω-oxidation and *O*-demethylation.

2.1.1.4. Rats

2.1.1.4.1. *In vivo* metabolism

Male Wistar rats previously cannulated received *in situ* gastrointestinal or intestinal perfusion with a mixture containing 2 mg *trans*-anethole/ml. Gastric absorption was 40.7% and intestinal absorption was 56.7% after 1 hour (Fritsch *et al.*, 1975). When rats were given a 100 mg/kg body weight oral dose of *trans*-[^{14}C]anethole, 20–30% of the radioactivity was present in the brain, liver, heart and stomach 6

*The radioactive carbon was located on the ring or in the C_3 position.

†The radioactive carbon was located on the ring.

Table 1. Interspecies data on the metabolism of *trans*-anethole

Species[1]/dose	*O*-Demethylation[2]	*O*-Demethylation metabolites (mg/kg body weight)	ω-Oxidation[3]	ω-Oxidation metabolites (mg/kg body weight)	Epoxidation[4]	Anethole epoxide (mg/kg body weight/day)	Reference
Human (M)							
1 mg (≈0.017 mg/kg body weight)	≈20%	3.4	≈59.5%	10	≈3%	0.510	Sangster *et al.*, 1987
Human (M & F)							
0.015 mg/kg body weight	≈13–17%	0.00195–0.00255	53–68%	0.00795–0.0102	≈3%[6]	0.000450	Caldwell and Sutton, 1988
0.87 mg/kg body weight	≈13–17%	0.113–0.148	53–68%	0.461–0.592	≈3%[6]	0.0261	Caldwell and Sutton, 1988
4 mg/kg body weight	≈13–17%	0.520–0.680	53–68%	2.12–2.72	≈3%[6]	0.120	Caldwell and Sutton, 1988
12 mg/kg body weight	NR[5]	NR	57%	6.84	≈3%[6]	0.360	Le Bourhis, 1973b
Mouse (M)							
50 mg/kg body weight[7]	≈47.3%	23.7	≈17.8%	8.90	≈6.2%	3.10	Sangster *et al.*, 1984a
62 mg/kg body weight ≈ 25%[8]	15.5	≈68%	42.2	≈7%	4.34	Bounds, 1994	
140 mg/kg body weight	≈26%[8]	36.4	≈68%	95.2	≈5%	7.00	Bounds, 1994
296 mg/kg body weight	≈23%[8]	68.1	≈72%	213	≈6%	17.8	Bounds, 1994
426 mg/kg body weight	≈20%[8]	107	≈74%	3152	≈7%	29.8	Bounds, 1994
Mouse (F)							
62 mg/kg body weight	≈24%[8]	14.9	≈68%	42.2	≈8%	4.96	Bounds, 1994
140 mg/kg body weight	≈21%[8]	29.4	≈72%	95.2	≈7%	9.80	Bounds, 1994
296 mg/kg body weight	≈22%[8]	65.1	≈70%	213	≈9%	26.6	Bounds, 1994
426 mg/kg body weight	≈25%[8]	107	≈66%	315	≈10%	42.6	Bounds, 1994
Mouse (M, F)							
0.05 mg/kg body weight[7]	≈71.8%	0.0359	≈10.4%	0.00520	≈1.6%	0.0008	Sangster *et al.*, 1984b
5 mg/kg body weight[7]	≈55.4%	2.80	≈15.3%	0.765	≈4.1%	0.205	Sangster *et al.*, 1984b
50 mg/kg body weight[7]	≈47.3%	23.7	≈18.3%	9.15	≈6.2%	3.10	Sangster *et al.*, 1984b
1500 mg/kg body weight[7]	≈34.6%	519	≈43.8%	657	≈8.8%	132	Sangster *et al.*, 1984b
Rat (M)							
100 mg/kg body weight	NR	NR	≈40%	40.0	NR	NR	Le Bourhis, 1970, 1973b
100 mg/kg body weight	≈32%	32.0	≈62%	62.0	NR	NR	Solheim and Scheline, 1973
101 mg/kg body weight	≈43%[8]	43.4	≈45%	45.5	≈12%	12.1	Bounds, 1994
254 mg/kg body weight	≈35%[8]	88.9	≈52%	132	≈13%	33.0	Bounds, 1994
500 mg/kg body weight	NR	NR	≈43%	215	NR	NR	Le Bourhis, 1970, 1973b
518 mg/kg body weight	≈33%[8]	171	≈52%	269	≈16%	82.9	Bounds, 1994
1039 mg/kg body weight	≈32%[8]	333	≈51%	530	≈20%	208	Bounds, 1994
Rat (F)							
0.05 mg/kg body weight	≈56.3%	0.0281	≈3.4%	0.00170	≈2.9%	0.00145	Sangster *et al.*, 1984b
5 mg/kg body weight	≈53.9%	2.69	≈8.9%	0.445	≈5.4%	0.270	Sangster *et al.*, 1984b
50 mg/kg body weight	≈41.8%	20.9	≈12.8%	6.40	≈14.3%	7.15	Sangster *et al.*, 1984a,b
101 mg/kg body weight	≈38%[8]	38.3	≈44%	44.4	≈18%	18.2	Bounds, 1994
254 mg/kg body weight	≈35%[8]	88.9	≈47%	119	≈18%	45.7	Bounds, 1994
518 mg/kg body weight	≈32%[8]	166	≈44%	228	≈23%	119	Bounds, 1994
1039 mg/kg body weight	≈26%[8]	270	≈49%	509	≈23%	239	Bounds, 1994
1500 mg/kg body weight	≈31.8%	477	≈22.4%	336	≈18.7%	281	Sangster *et al.*, 1984b

[1]M = male; F = female. [2]Measured as expired CO_2. [3]Measured as total %. Possible metabolites reported include *p*-methoxybenzoic acid, *cis-p*-methoxycinnamic acid, *trans-p*-methoxycinnamic acid, *p*-methoxyhippuric acid, *p*-methoxyacetophenone, *p*-methoxycinnamoylglycine, *p*-hydroxybenzoic acid, *p*-hydroxyhippuric acid, *p*-hydroxybenzaldehyde, *p*-hydroxyphenylacetic acid and *p*-hydroxycinnamic acid. [4]Measured as total percentage of diastereomer mixture of diol and mercaptan. [5]NR = not reported. [6]The % epoxidation was not actually measured but is estimated here based on other reported data (Sangster *et al.*, 1987). [7]Doses were administered by ip injection. [8]Measured as total *O*-demethylation metabolites following administration of *trans*-[^{14}C]anethole.

hours later. After 48 hours, greater than 90% of the radioactivity was present in the urine with less than 1% in the faeces or body (Strolin-Benedetti and Le Bourhis, 1972).

Approximately 70% of a 100 mg [^{14}C]anethole/kg body weight dose administered as an aqueous emulsion in 1% Tween-20 to each of 18 male rats (strain not specified) was detected in the body immediately after dosing, 25% after 3 hours, and 0% after 10 hours. Maximum blood levels of 7 mg/litre, reached 10 minutes after the dose, decreased rapidly to 2 mg/litre over the next hour. Audioradiography performed 3 hours after dosing with anethole revealed most radioactivity in the liver and intestine. Urinary metabolites included glycine conjugates of ω-oxidation, *O*-demethylation, and combined ω-oxidation and *O*-demethylation products (*p*-methoxybenzoic acid, 34%; and *p*-hydroxybenzoic acid, 6%). A 500 mg/kg body weight oral dose administered to three rats gave the same urinary metabolites within 24 hours including *p*-methoxybenzoic acid (39%) and *p*-hydroxybenzoic acid (5%) in the form of sulfate (28%) and glycine (14%) conjugates (Le Bourhis, 1970, 1973b).

Male albino rats were given a single oral dose of 100 or 400 mg *trans*-anethole/kg body weight. Urine was collected 48 hours later and was treated with β-glucuronidase and sulfatase. Urinary metabolites which accounted for essentially all of the dose included ω-oxidation metabolites (mainly *p*-methoxyhippuric, *p*-methoxycinnamic acid and *p*-methoxybenzoic acid), *O*-demethylation metabolites (mainly *p*-hydroxypropenylbenzene) and metabolites derived from combined pathways (such as *p*-hydroxycinnamic acid). At the 400 mg/kg body weight dose level, trace amounts of the epoxidation metabolites [i.e. 1-(*p*-methoxyphenyl)-1,2-propanediol] also were detected. Biliary metabolites exhibited a similar metabolic composition, but contained more of the epoxidation metabolite (diol) and trace quantities of intermediary metabolites (e.g. *p*-methoxycinnamaldehyde) (Solheim and Scheline, 1973).

In a parallel study, similar urinary and biliary metabolic profiles were observed when male albino Wistar rats (five/dose) were given a single oral dose of 200 or 400 mg 3,4-dimethoxypropenylbenzene (isoeugenyl methyl ether)/kg body weight. Principal bile metabolites included those formed by 3- and 4-*O*-demethylation and ω-oxidation (3,4-dimethoxycinnamic acid and glycine conjugate). Greater than 77% of urinary metabolites were formed by a combination of *O*-demethylation and ω-oxidation and consisted of mainly 4-hydroxy-3-methoxycinnamic acid and the glycine conjugates of 3,4-dimethoxybenzoic acid and 3,4-dimethoxycinnamic acid. Epoxidation metabolites accounted for less than 1% of the urinary metabolites (Solheim and Scheline, 1976).

In recent studies, the metabolism of *trans*-anethole has been more accurately quantified. Female Wistar rats were administered a single oral dose of 50 mg *trans*-[methoxy-^{14}C]anethole/kg body weight. 10 urinary metabolites which accounted for 84% of the dose included mainly *O*-demethylation (41.8%) and ω-oxidation (12.8%) metabolites. The epoxidation pathway in the rat accounted for approximately 14.3% of the urinary metabolites, which was more pronounced than in the mouse (Sangster *et al.*, 1984a) or in humans (Caldwell and Sutton, 1988; Sangster *et al.*, 1987).

The rate of elimination of an ip dose of 250 mg *trans*-[$^{14}C_1$]anethole/kg body weight was slower for female rats compared with male rats. In males, 71% of the radioactivity was recovered from the urine after 24 hours with an additional 6% recovered after 48 hours. In females, 56% of the radiolabel was recovered from the urine after 24 hours with an additional 15% recovered after 48 hours (Caldwell *et al.*, 1991a,b).

The effect of dose level on metabolism was studied in female Wistar rats (four/group) administered a single dose of 0.05, 5, 50 or 1500 mg *trans*-[methoxy-^{14}C]anethole/kg body weight. A shift in the metabolic continuum was observed from *O*-demethylation at the low dose (56% at 0.05 mg/kg body weight to 32% at 1500 mg/kg body weight) to side-chain ω-oxidation (e.g. 2.6% free and glycine conjugated *p*-methoxybenzoic acid at 0.05 mg/kg body weight and 17.5% at 1500 mg/kg body weight) and epoxidation (3% at 0.05 mg/kg body weight to 18% at 1500 mg/kg body weight) at high doses (Sangster *et al.*, 1984b).

These results were confirmed in Sprague–Dawley CD rats (six/group/sex) pre-fed *trans*-anethole for 3 weeks at levels resulting in an average daily intake of 0, 100, 250, 500 or 1000 mg/kg body weight. As in the mouse study, a control group was maintained for each of the four groups of rats that received *trans*-anethole for 21 days. All groups of control and pre-fed rats were then given a single oral dose of *trans*-[$^{14}C_1$]anethole by gavage at a dose equivalent to the pre-feeding level determined during week 3 of pre-feeding. The rate of elimination of urinary metabolites after 24 hours decreased significantly with increasing dose in control animals (i.e. non-pre-fed rats) (80% at 100 mg/kg body weight *v.* 50% at 1000 mg/kg body weight), but was essentially constant for all pre-fed groups ($>80\%$). These data suggest that pre-feeding increases the rats' capacity to metabolize and eliminate doses of *trans*-anethole (Bounds, 1994; Bounds and Caldwell, 1992, 1996).

The importance of the *O*-demethylation pathway decreased with increasing dose, particularly in female rats, while epoxidation (i.e. diol) and ω-oxidation metabolites increased with dose independent of sex or pre-feeding. Quantitatively, epoxidation accounted for more of the administered dose in the female. The proportion of GSH conjugation of the epoxide did not vary significantly with dose, but the

female excreted significantly more than the male, suggesting that the other detoxication pathway (epoxide hydrolase) is less important in the female (Bounds and Caldwell, 1992, 1996).

It may be postulated that the extent of epoxidation may not be adequately represented by levels of diol and GSH-derived metabolites detected in the urine. That is, AE and AE metabolites (i.e. diol or GSH metabolites) may be further oxidized and cleaved to yield other urinary metabolites. However, metabolic data from dose-dependent studies of *trans*-anethole and from other studies of structurally related substances in rats do not support this postulate. First, an increase in excreted AE metabolites in the rat at high dose levels is not accompanied by an increase in metabolites from side-chain oxidation (Sangster *et al.*, 1984a,b). Secondly, rapid conjugation of diols by glucuronosyl transferase (Kasper and Henton, 1982) permits little opportunity for CYP450-catalysed oxidation of the terminal methyl group of the diol metabolite. It is also unlikely that ω-oxidation metabolites undergo epoxidation, particularly the carboxylic acids, which are readily conjugated with glycine. The absence of even trace quantities of epoxidation-type metabolites (1,2-diols and 1,2-thioalcohols from GSH conjugation) in the urine of rats given 4-methoxycinnamic aldehyde (Solheim and Scheline, 1973), 3,4-dimethoxycinnamic acid (Solheim and Scheline, 1976) and cinnamaldehyde (Peters and Caldwell, 1994) provides evidence that epoxidation of ω-oxidation metabolites does not occur. Therefore, it is likely that little if any crossover occurs between these different metabolic pathways, and the relative quantities of diol and GSH metabolites detected in the urine reasonably reflect the extent of epoxidation of *trans*-anethole. The data clearly show that in the rat, the pattern of *trans*-anethole metabolism is dose dependent and, in particular, there is a metabolic shift to epoxidation at higher dose levels.

2.1.1.4.2. Enzyme induction studies

The results of metabolism studies suggesting that AE is the presumed hepatotoxin in the rat fostered numerous enzyme induction and *in vitro* metabolism studies. Male Wistar rats (three/group) given 10 daily doses of 125 and 250 mg *trans*-anethole/kg body weight by gavage resulted in an increase in relative liver weight at the 250 mg/kg body weight level. Liver cytochrome P450 content was unaffected by treatment and only marginal* increases were observed in ethoxyresorufin *O*-deethylation (EROD) and pentoxyresorufin *O*-dependentylation (PROD) activities, markers for the induction of CYP1A and CYP2B family of CYP450 isoenzymes, respectively (Burke and Mayer, 1974; Burke *et al.*, 1977; Lubet *et al.*, 1991). Significant† increases in Phase II enzyme activities [i.e. glucuronosyl transferase, glutathione *S*-transferase (GST) and diaphorase] were observed at the 250 mg/kg body weight dose level suggesting that increased detoxication could occur via conjugation (Rompelburg *et al.*, *1993)*.

Confirmation of the results observed in the enzyme induction studies was obtained when Sprague–Dawley CD rats (eight/sex/group) were maintained on diets containing 0, 0.25, 0.5 and 1.0% *trans*-anethole for 21 days. A dose-related increase in relative liver weight, total CYP450 content and microsomal protein was reported indicating a response to enzyme induction and increased capacity to metabolize *trans*-anethole. Marginal increases in the activities of EROD and PROD were considered by the authors to indicate minimal induction of CYP450. Sprague–Dawley CD rats (24) were given seven daily ip doses of 300 mg *trans*-anethole/kg body weight or one of the model CYP450 inducers β-naphthoflavone (BNF), phenobarbitione (PB) or isosafrole (ISF). *trans*-Anethole-treated rats exhibited increases in relative liver weight, microsomal protein and microsomal CYP450 (45%). Other CYP450 inducers exhibited more significant increases in microsomal CYP450 activity (96% for BNF, 113% for PB and 133% for ISF). Induction of CYP450-related activity of 7-ethoxycoumarin deethylase (ECOD) was significantly greater for isosafrole (592% of controls) than for *trans*-anethole (69%) (Reed and Caldwell, 1992a). *trans*-Anethole did not induce dealkylase enzymes, suggesting that metabolic switching from *O*-demethylation to side-chain oxidation and epoxidation at high dose levels is the result of saturation of demethylation enzymes.

The pattern of induction of Phase I (oxidation-reduction metabolism) and II (conjugation) enzymes by *trans*-anethole is not indicative of receptor-mediated induction by aryl hydrocarbons. In receptor-mediated induction, very low dose levels (e.g. 0.001 mg/kg) of potent aryl hydrocarbon inducers such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) bind to a cytosolic aryl hydrocarbon (Ah) receptor protein which associates with specific DNA sequences and migrates into the nucleus. The receptor-inducer complex acts on regulatory sites on selective genes resulting in synthesis of mainly the CYP4501A family of monooxygenase isoenzymes, specifically aryl hydrocarbon hydroxylase, and other non-monooxygenase enzymes (e.g. glucuronosyl transferase) (Okey, 1990). This type of induction shows strain and species sensitivity and does not result in a marked proliferation of smooth endoplasmic reticulum. *trans*-Anethole acts like a weak phenobarbital-type inducer which induces a broad range of monooxygenase and conjugation enzymes

*The increase was less than twofold as compared to greater than 50-fold for Aroclor 1254 (Luber *et al.*, 1991).

†The increase was greater than twofold, approaching the threefold change observed for maximum induction.

at much higher doses concurrent with proliferation of smooth endoplasmic reticulum and, after prolonged exposure, increased liver weight (Hodgson, 1994).

2.1.1.4.3. *In vitro* metabolism and related genotoxicity assays

The dose-dependent increase in AE formation has been correlated to the observed cytotoxicity exhibited by *trans*-anethole in the rat (see Section 2.2.3.2.). Dose-dependent cytotoxicity [as measured by lactate dehydrogenase (LDH) leakage] and cellular GSH depletion are observed when freshly prepared hepatocytes derived from female Sprague–Dawley CD rats are incubated with *trans*-anethole. Some cytotoxicity was observed at a concentration of 1×10^{-3} M, while a concentration of 5×10^{-3} M produced clear evidence of cytotoxicity.

To evaluate the cytotoxic effects from increased levels of AE, *trans*-anethole was assayed in the presence of cytosolic (4-fluorochalcone) and microsomal (e.g. trichloropropylene oxide) inhibitors of epoxide hydrolase (EH). Cytosolic EH inhibition caused a rapid (1 hour) increase in cytotoxicity at sub-cytotoxic levels (5×10^{-4} M) of *trans*-anethole, while microsomal EH inhibition had no effect. Pretreatment with GSH depletors (e.g. diethyl maleate) resulted in delayed (4 hours) cytotoxicity suggesting that detoxication of the epoxide by epoxide hydrolase is more efficient than GST. Results of these studies confirm that *trans*-anethole epoxide is cytotoxic to rat hepatocytes, but only when detoxication pathways (i.e. "rapid" hydration by cytosolic EH or "slow" GSH conjugation) are saturated by either high dose levels of *trans*-anethole or by metabolic inhibitors (Marshall and Caldwell, 1992).

In a subsequent study, cytotoxicity, as measured by leakage of cytosolic LDH from hepatocytes of male and female SD-CD rats, was observed at concentrations of 10^{-3} M or more for *trans*-anethole and 10^{-4} M or more for AE (Marshall and Caldwell, 1996). In a standard *in vitro* unscheduled DNA synthesis (UDS) assay using hepatocytes from female Sprague–Dawley CD rats, no evidence of genotoxicity was exhibited by either *trans*-anethole or *trans*-anethole epoxide at cytotoxic concentrations (i.e. $\geqslant 10^{-3}$ M or $\geqslant 10^{-4}$ M, respectively). Inhibition of the epoxide hydrolase detoxication pathway increased the cytotoxic response, but failed to induce UDS in hepatocyes (Caldwell *et al.*, 1992; Howes *et al.*, 1990a; Marshall and Caldwell, 1992, 1996; Marshall *et al.*, 1989). No UDS was detected in the hepatocytes isolated from female rats given a single oral dose of 0, 1, 125 or 500 mg *trans*-anethole/kg body weight (Marshall and Caldwell, 1996).

Taken together, the *in vivo* metabolism data and the results of *in vitro* studies designed to investigate the hepatotoxic potential of AE support the conclusion that at high dose levels of *trans*-anethole, a significant increase in the epoxidation pathway occurs yielding hepatocellular concentrations ($> 10^{-4}$ M) of *trans*-anethole epoxide sufficient to saturate available detoxication pathways (e.g. cytosolic hydration by EH) and induce cytotoxicity in hepatocytes of male and female rats.

2.1.2. Summary of metabolic and biochemical data

A summary of data comparing the metabolic fate of *trans*-anethole in the mouse, rat and human is presented in Table 1. At all dose levels tested, rodents and humans used a combination of detoxication pathways (*O*-demethylation and ω-oxidation) and an intoxication pathway (epoxidation) to metabolize *trans*-anethole. At low dose levels (1–5 mg/kg body weight), rodents detoxicate *trans*-anethole primarily via *O*-demethylation, which is favoured about equally in the mouse (55–72%) and rat (54–56%). In humans, detoxication at low dose levels (0.015–12 mg/kg body weight) occurs primarily via the ω-oxidation pathway (53–68%). In all three species, intoxication via epoxidation is a minor pathway ($< 6\%$) in the low dose range, with the rat showing a slightly greater tendency to epoxidize compared with the mouse or human (mouse 1.6–4.1%; rat 2.9–5.4%; human approx. 3%). These data support the conclusion that at low dose levels (< 12 mg/kg body weight) daily production of AE is independent of species and in all cases represents a minor metabolite.

As *trans*-anethole dose levels increase in the range of 100 to 200 mg/kg body weight, a metabolic shift is observed in rodents. *O*-demethylation decreases while ω-oxidation becomes the primary pathway in both mice and rats, and epoxidation becomes significant in rats. In both sexes of mice, *O*-demethylation decreases from approximately 55% at 5 mg *trans*-anethole/kg body weight/day to 21–26% at 140 mg/kg body weight/day. In the same dose range, ω-oxidation increases to approximately 70% from approximately 15%, while epoxidation remains essentially constant (i.e. approx. 5–7% from 4%). The shift to ω-oxidation is associated with weak phenobarbital-type induction of CYP-450 enzymes (Hodgson, 1994) (see Section 2.1.1.4.2.). It has been suggested (Newberne, 1997) that the continuous induction of the ω-oxidation pathway in mice may result in hepatocellular and organ weight changes similar to those observed in a mouse 90-day toxicity study (see Section 2.2.2.1. and 2.2.3.1.).

In both sexes of rats, ω-oxidation increases to approximately 45% at high dose levels (> 100 mg/kg body weight) from 3–15% at low dose levels ($\leqslant 50$ mg/kg body weight). More significantly, the epoxidation pathway increases to approximately 12–20% in males and to approximately 23% in females at high dose levels (> 100 mg/kg body weight/day) from the 3–5% range at low dose levels ($\leqslant 5$ mg/kg body weight). Increased epoxidation

Table 2. Acute toxicity studies *trans*-anethole

Species	Route*	LD_{50} (mg/kg body weight)	Reference
Mouse	oral	1820–5000	Levenstein, 1960; Jenner *et al.*, 1964; Boissier *et al.*, 1967
Mouse	ip	1410–650	Caujolle and Meynier, 1958; Boissier *et al.*, 1967
Rat	oral	2090–3200	Shelanski and Gabriel, 1958b; Jenner *et al.*, 1964; Taylor *et al.*, 1964; Boissier *et al.*, 1967
Rat	ip	2670–900	Caujolle and Meynier, 1958; Boissier *et al.*, 1967

*ip = ip injection.

may lead to higher hepatocellular concentrations of AE, which would be expected to saturate available "fast" Phase I (epoxide hydrolysis) and "slow" Phase II (glutathione conjugation) detoxication pathways (Marshall and Caldwell, 1996). Lower cytosolic EH activity may lead to higher hepatocellular concentrations of AE in the female. Continuously high hepatocellular concentrations of *trans*-anethole epoxide are, in all probability, linked to chronic cytotoxicity, hyperplasia of hepatocytes, and subsequent cellular alterations leading to hepatocarcinogenicity in female rats (see Section 2.2.3.2.). The observation that the rat converts *trans*-anethole to anethole epoxide to a greater extent than the mouse (or the human) is consistent with the increased susceptibility of the rat to *trans*-anethole exposure as reflected by the lower NOAELs (see Table 3 and Section 2.2). Thus, the rat is considered the most sensitive species for the purpose of evaluating the potential for toxicity in humans exposed to *trans*-anethole from use as a flavouring substance (see Section 4.0).

2.2. Toxicological studies

2.2.1. Acute toxicity

The results of oral acute toxicity studies for *trans*-anethole are summarized in Table 2. Oral LD_{50} values for *trans*-anethole are in the range from 1820 to 5000 mg/kg body weight. For 4 days, Osborne–Mendel or Sherman rats (three/sex) were maintained on diets containing *trans*-anethole at levels calculated to result in an average daily intake of 695 mg/kg body weight. Mild liver changes were reported including slight discoloration, mottling and blunting of the lobe edges (Taylor *et al.*, 1964).

2.2.2. Short-term toxicity studies

2.2.2.1. Mice

To evaluate the dose tolerance of orally-administered *trans*-anethole, six groups (five/sex/ group) of CD-1 mice were given anethole in the diet at target dose levels of 0, 60, 120, 240, 360 or 500 mg/kg body weight/day for 28 days (Minnema, 1997). Dietary levels for the three highest dose groups were achieved in a stepwise fashion. The addition of anethole to the diet resulted in a palatability problem for the mice administered the higher dose levels, resulting in decreased food intake and decreased body weight gain. There were no histopathologic changes that were considered treatment related, but other factors such as reduced food intake and decreased body weight gain were used to establish doses in the 90-day study (Minnema, 1997).

Five groups (20/sex/group) of CD-1 mice were given anethole in the diet at target dose levels of 0, 30, 60, 120 or 240 mg/kg body weight/day for 90 days (Minnema, 1997). The target dose levels were achieved using a stepwise dose elevation process during the first 2 weeks of the study to alleviate the potential for diminishing palatability of the test substance. Increased mortality was observed at dietary levels 60 mg/kg or more body weight/day in males and 120 mg/kg or more body weight in females and was attributed to "inanition syndrome" characterized by decreases in food consumption, water intake and physical activity, presumably associated with palatability of the diets. The pattern of body weight changes and food consumption data supported this conclusion. Body weights recorded biweekly for 5 weeks and then decreased weekly at levels 120 mg/kg or more body weight/day in males and at 240 mg/kg body weight/day in females. Daily food consumption decreased at levels 120 mg/kg or more body weight/day in both males and females. In males, decreased consumption was accompanied by decreased food efficiency uptake with a particularly marked effect observed at weeks 3 and 4, when dietary levels were increased to 240 mg/kg body weight/day. Gross and histopathological evaluation and organ weight measurements performed at week 13 provided additional evidence of inanition. Reduction in liver glycogen content was observed at levels 30 mg/kg body weight or more/day in males and at 60 mg/kg or more body weight/day in females. Poor growth resulted in reduced kidney to brain weight and spleen to brain weight in males at the highest dietary level. The latter effect was correlated with decreased cellularity.

Histopathologic examination revealed an increase in hepatocellular hypertrophy in males at levels 60 mg/kg or more body weight/day, and enlarged livers and a dose-dependent increase in relative liver weights in all groups of treated male mice. These

Table 3. Incidence of hepatic effects observed in rodent oral toxicity studies of *trans*-anethole

Species	Duration (days)	Sex	Dose (mg/kg body weight/day)	1	2	3	4	5	6	Ref
Rat	90	F	150							A
Rat	90	F	300							A
Rat	90	F	600	(14/20)†,§						A
Rat	90	F	900	(20/20)†,§	(10/20)‡,§	(10/20)‡,§				A
Rat	90	M	150							A
Rat	90	M	300	(20/20)†,§						A
Rat	90	M	600	(20/20)†,§	(9/20)‡,§					A
Rat	90	M	900	(20/20)†,§	(14/20)‡,§	(13/20)‡,§				A
Rat	819–847	F	120							B
Rat	819–847	F	250	(9/52)††			(16/52)**			B
Rat	819–847	F	550	(12/52)††			(29/52)††	(19/52)**	(6/52)**	B
Rat	819–847	M	100							B
Rat	819–847	M	200					(7/52)§		B
Rat	819–847	M	400				(32/52)††	(16/52)††		B
Mouse	90	F	30, 60, 120, 240							A
Mouse	90	M	30							A
Mouse	90	M	60	(4/17)†,§						A
Mouse	90	M	120	(10/18)†,§						A
Mouse	90	M	240	(12/17)†,§						A

†The author considered hepatocellular hypertrophy reported in both sexes of rats and male mice treated with *trans*-anethole to be an adaptive physiological response associated with the known modest enzyme inducing (Reed and Caldwell, 1993) properties of *trans*-anethole (Minnema, 1997), and as such is not an adverse event (Newberne, 1997).

‡Reported by the study author. In an independent evaluation (Newberne, 1997), statistical significance could not be confirmed.

§$P < 0.05$.

**$P < 0.01$.

††$P < 0.001$.

[1]Hepatocellular hypertrophy.

[2]Hepatocellular individual cell necrosis.

[3]Pigmented macrophages in perivascular inflammatory infiltrate.

[4]Sinusoidal dilation.

[5]Nodular hyperplasia.

[6]Hepatocellular carcinoma.

[A]Minnema, 1997.

[B]Truhaut *et al.*, 1989.

treatment related effects were considered to be adaptive physiological responses resulting from the known enzyme-inducing properties of *trans*-anethole. No significant difference was observed between any group of treated females and controls. Blood chemical determinations revealed a statisically insignificant increase in alanine and aspartate transaminase values at levels 120 mg/kg or more body weight/day which, in conjunction with hepatocyte hypertropy, suggests mild enzyme induction. A statistically significant ($\leqslant P = 0.05$) increase in alkaline phosphatase (approx. $\approx 2 \times$ mean values for controls) at levels 120 mg/kg or more body weight/day was not accompanied by histological evidence of toxicity; increased blood alkaline phosphatase values have been reported to occur in rats maintained on food restricted diets for 90 days (Schwartz *et al.*, 1973). With the exception of reduced hepatocellular glycogen, there was no evidence of abnormalities to the livers of female mice (Minnema, 1997) (see Table 3). Considering the lack of any evidence of histopathology, the minimal changes observed in serum values for phosphatase and aminotransferase enzymes are, in all probability, responses to the unpalatability and the need to metabolize anethole. Based on the lack of treatment-related effects observed at the dose levels administered, the author assigned a NOAEL of more than 240 mg/kg body weight/day.

An independent histopathological evaluation (Newberne, 1997) was performed and revealed the presence of several direct and indirect treatment-related effects. A variable but significant increase in hepatocellular hypertrophy was observed in all treatment groups of male and female mice, with increased grade of involvement at higher doses. Intensity was greatest in the highest dose group of each sex, but non-dose related in the other female groups. To provide a more objective basis for interpreting the hepatocellular hypertrophy data, the degree of associated hepatocyte nuclear pleomorphism observed in both control and test animals was assessed.

Hepatocyte nuclear pleomorphism is recognized as a common morphologic entity in normal, untreated mammalian livers but may be associated with potential toxicity under some conditions. There were no differences in the incidence of nuclear pleomorphism between treated and control groups, and only the males in the highest dose group exhibited a statistically higher value (i.e. compared with control mean value) for intensity suggesting that this type of response is not significant in mice exposed to *trans*-anethole. Decreased hepatocyte glycogen content, based on the pattern

and extent of the finely to coarsely vacuolated perinuclear cytoplasmic clearing in stained liver sections, was observed in all treated groups of both sexes and was statistically significant as compared to controls in all groups except low-dose females. This effect may be associated with decreased palatability of diet and food intake or the failure to fast the mice in a uniform fashion immediately prior to sacrifice; it is not considered a pathologic alteration.

Increased enzyme induction is associated with increased liver protein synthesis and, thus, real or potential increased liver weight. The increased serum enzyme concentrations observed in some treated groups of the anethole study suggest some minor injury to the liver, but not of the magnitude necessary to induce histopathological lesions. The presence of hepatocyte hypertrophy in all treated groups of both sexes, the increases in absolute and relative liver weights in higher dose males, and the absence of any other significant abnormality on histopathologic examination, mutually support the conclusion that the hepatocyte hypertrophy is the result of hepatic enzyme induction related to an increased requirement for *trans*-anethole metabolism. The histomorphologic observations associated with *trans*-anethole exposure in mice were concluded to be adaptive and physiologic but not pathologic. Under conditions of this study, there were no treatment-related adverse effects observed. Therefore, the NOAEL was concluded to be more than 240 mg/kg body weight/day in both male and female CD-1 mice (Newberne, 1997). These results are consistent with the results of the aforementioned enzyme induction studies in rats (see Section 2.1.1.4.2.) and the known metabolic fate of *trans*-anethole in mice in which dose levels 62 or more mg *trans*-anethole/kg or more body weight are mainly metabolized by CYP-450 catalysed ω-oxidation (see Section 2.1.1.3.).

In a study using 4-day-old CD-1 male and female mice, *trans*-anethole was administered twice weekly for 5 weeks at dose levels of 0, 450 or 900 mg/kg body weight and observed until necropsy at 14 months. There was no statistically significant increase in hepatomas observed in treated animals as compared with controls. However, males in both treated and control groups developed hepatomas in slightly higher incidence than females (Miller *et al.*, 1983). Based on the duration of the study (5 weeks), this study is considered inadequate for evaluating chronic toxicity.

2.2.2.2. Rats

Male and female rats were administered orally 0, 0.1, 0.3, 1.0 or 3.0% *trans*-anethole in the diet for 90 days. The corresponding dose levels are approximated to be 0, 50, 150, 500 and 1500 mg/kg body weight/day, respectively. Increasingly severe degeneration and regeneration of the hepatic cells and hepatic cell oedema were reported at dose levels of 150 mg/kg body weight/day (Shelanski, 1958a).

Two groups of 10 Wistar male and female rats each were administered *trans*-anethole in their drinking water for 3 months at a dietary level of 11.4 mg/kg body weight/day (Vignoli *et al.*, 1965). The first and second groups were comprised of adult and weanling rats, respectively. Electrocardiograms and electroencephalograms were reported as normal for both groups. No adverse effects related to body weight, gross pathology or histopathological examination of the liver and brain were observed in either group.

trans-Anethole was administered in the diet to groups of five male and five female Osborne–Mendel rats providing daily dose levels of 2500 or 10,000 ppm for 52 weeks or 15 weeks, respectively. The corresponding dose levels were calculated (FDA, 1993) to be 125 or 500 mg *trans*-anethole/kg body weight/day. Control groups contained five males/five females or 10 males/10 females, respectively. Slight hydropic changes of the hepatic cells were reported in males at the highest dose level (Hagan *et al.*, 1967). At the 125 mg *trans*-anethole/kg body weight/day dose level, no effects were reported on growth or haematology, and no macroscopic or microscopic changes were observed in the tissues. The dose level of 125 mg/kg body weight/day that resulted in no adverse effects is more than 1000 times the estimated daily *per capita* intake* ("eaters only") of 54 μg/kg body weight from use of *trans*-anethole as a flavouring substance.

Five groups (20/sex/group) of Sprague–Dawley rats were given anethole in the diet at target dose levels of 0, 150, 300, 600 or 900 mg/kg body weight/day for 90 days (Minnema, 1997). The target dose levels were achieved using a stepwise dose elevation process during the first 2 weeks of the study to alleviate the potential for diminishing palatability of the test substance. Weekly measurement of body weights (biweekly for the first 4 weeks), daily measurement of food consumption, and weekly calculation of efficiency of food utilization all revealed decreased values at doses 300 mg/kg or more body weight/day in males and 600 mg/kg or more body weight in females, changes not considered treatment related by the authors, but rather related to the reduced palatability of the diet. The authors reported mild but significant increases in liver-to-

*The daily *per capita* intake ("eaters only") is a method adopted by the US Food and Drug Administration (FDA) to estimate "high" intake eaters. It is calculated as follows: μg/kg body weight = (annual volume, kg) $\times$ $(1 \times 10^9$ μg/kg) $\times$ $(1/260 \times 10^6$ people in the US) $\times$ (1/365 days) $\times$ (1/60 kg body weight) $\times$ (1/0.6) $\times$ 10 (eaters only) and is based on the assumptions that (1) only 10% of the population, the "eaters only", consumed the entire reported annual volume of a flavouring substance (NAS, 1987), and (2) only 60% of the flavour volume was reported by flavour manufacturers in the annual survey (NAS, 1987).

Table 4. Natural occurrence of *trans*-anethole

Substance/food item	Concentration in volatile fraction (ppm)*
trans-Anethole:	
Anise (*Pimpinella anisum L.*)	752,000–961,000
Vegetable fennel [var. azoricum (Miller)]	734,800–911,300
Sweet fennel [var. dulce (Miller)]	581,200–906,700
Bitter fennel [var. vulgare (Miller)]	213,600–895,200
Illicium verum	88,000–910,000
Illicium anisatum	12,000
Angelica root oil	8,900
Thymus vulgaris I.	5,600
Cinnamon (*Cinnamomum zeylanicum Blume*)	3200
Ouzo	115–181
Clove bud	100
Nutmeg (*Myr. frag. Houttuyn*), *Thymus zygis I.*	trace
Pepper (*Piper nigrum L.*), coriander seed (*Coriandrum sativum L.*), dill seed (*A. graveolens L.*), Indian dill herb (*A. sowa. Roxb.*), *Ocimum basilicum* varieties	Quantity not reported

*CIVO-TNO, 1994.

body-weight ratios in the rats at dose levels 300 mg/kg or more body weight/day. Centrilobular to diffuse hepatocellular hypertrophy was reported at dose levels of 300 mg/kg or more body weight/day in males and 600 mg/kg or more body weight/day in females. Blood chemistry analyses revealed increases in γ-glutamyltransferase at target doses of 900 mg/kg body weight/day in males and 600 mg/kg or more body weight in females and in alanine and aspartate aminotransferase values at 900 mg/kg body weight/day in females. The authors considered the increase in liver-to-body-weight ratios, hepatocellular hypertrophy, serum alanine and serum aspartate aminotransferase values to be adaptive physiological responses associated with the enzyme induction properties (Reed and Caldwell, 1993) of *trans*-anethole and, therefore, were not considered to be adverse effects (see Table 3).

Minimal to slight single cell hepatocellular necrosis, associated with perivascular inflammatory infiltrate was recorded in rats at dose levels 600 mg/kg or more body weight/day in males and 900 mg/kg body weight in females (see Table 3). In both sexes at the highest intake level (900 mg/kg body weight/day), the incidence of pigment associated with perivascular infiltrate was increased. Based on the observed necrosis in males and increased levels of γ-glutamyltransferase in females, the NOAEL was reported to be 300 mg *trans*-anethole/kg body weight/day (Minnema, 1997).

An independent histopathological evaluation on the livers of the above referenced rats (Newberne, 1997) revealed a statistically significant increase in hepatocellular hypertropy at dietary levels 300 mg/kg or more body weight/day in males and 600 mg/kg or more body weight/day in females. The linear increased incidence and degree of involvement of this change was typical, morphologically, of a weak phenobarbital-type enzyme induction (Hodgson, 1994) resulting from exposure to any one of a number of chemicals, including *trans*-anethole. High dose levels of *trans*-anethole act as low potency inducers and cause a marked proliferation of the smooth endoplasmic reticulum (SER) and some induction of CYP450 Phase I and Phase II enzymes. No significant strain and species differences appear to exist in laboratory animals for this type of induction, although it may be more pronounced in the mouse. Continuous exposure leads to a morphologic continuum of proliferation of SER, hypertrophy and increased liver weights. This is an adaptive change, not a lesion, and as such is not an adverse event (Newberne, 1997).

Hepatocellular necrosis (focal or single cell, mononuclear infiltrates, pigmented macrophages) was present in some rats of all groups of treated and control animals. However, in the independent evaluation, neither the incidence or extent of involvement these areas was sufficient to conclude a relationship to anethole exposure (Newberne, 1997). This is contrary to that reported by the authors of the study (Minnema, 1997). Serum chemistry values were elevated in some treated animals, but were not clearly different from controls*. Furthermore, they were not supported by hepatocellular histopathology. Decreased glycogen of the hepatocytes occurred in all treated male and female rats but was not dose related and, as noted above, is probably a result of the unpalatable nature of the diet and/or decreased food intake (Newberne, 1997). Under conditions of the study (Minnema, 1997), there were no treatment-related histopathological adverse effects attributable to *trans*-anethole exposure. Therefore, it was concluded that the NOAEL

*Occasional individual values in control animals were comparable to or higher that those of treated animals. However, when these higher individual values were combined with other control values, they were no longer meaningful. This suggests that although the means of some treated groups tended to be elevated, their significance is not clearly related to anethole exposure (Newberne, 1997).

would be more than 900 mg/kg body weight/day (Newberne, 1997).

The conclusion by Newberne that *trans*-anethole does not produce hepatotoxicity in rats after 90 days is supported by the lack of hepatotoxic effects observed in a stop-exposure group of rats provided dose levels of 400 or 550 mg *trans*-anethole/kg body weight/day (males or females, respectively) for 54 weeks in a carcinogenicity study (Truhaut *et al.*, 1989) (see Section 2.2.3.2.).

2.2.2.3. Rabbits

10 adult male rabbits were given *trans*-anethole in their drinking water for 3 months at a dietary level of 11.4 mg/kg body weight/day. Body weight gain was not affected by the test substance. Gross pathology and histopathological examinations of the liver and brain were normal on all animals. Electrocardiograms and electroencephalograms were reported as normal (Vignoli *et al.*, 1965).

2.2.3. Long-term toxicity studies/carcinogenicity studies

2.2.3.1. Mice

In the first of a series of experiments conducted to investigate the carcinogenic potential of *trans*-anethole in CD-1 mice, groups of either 56 to 67 males or 55 to 61 females were administered *trans*-anethole via gavage at doses of either 370 or 740 mg/kg body weight twice weekly for 10 weeks. The animals were sacrificed at 11 to 14 months and evaluated for the incidence of hepatomas. In a second experiment, 53 male CD-1 mice were administered 703 or 1390 μg *trans*-anethole/mouse by ip injection on days 1, 8, 15 and 22 after birth. The animals receiving the lower dose were sacrificed after 13–18 months, and the animals from the higher dose group were sacrificed after 12 months. In a third experiment, groups of female CD-1 mice were administered 690 mg *trans*-anethole/kg body weight/day in the diet for 12 months followed by a 6-month period in which the animals received only the basal diet, after which they were sacrificed. In the fourth and final experiment, a group of 17 female A/J mice was administered *trans*-anethole via ip injection twice weekly for 12 weeks at a dose level of 148 mg/kg body weight, and sacrificed at 8 months.

At necropsy in each of these four experiments, there was no increase in the incidence of hepatic tumours in anethole-treated mice when compared with the controls (Miller *et al.*, 1983). However, for the purpose of evaluating carcinogenicity, these studies in mice are judged to be inadequate when compared to the design and conduct of a current standard 2-year bioassay.

Groups of 20 A/He female mice were administered, three times weekly by ip injection, 2, 4 or 12 mg anethole/kg body weight for 8 weeks. The liver, kidney, spleen, thymus, intestine and salivary and endocrine glands were examined macroscopically for tumours. No increase in tumour incidence was reported at any dose level. Survival of all groups was reduced to approximately 70% by the end of the study period (Stoner *et al.*, 1973).

2.2.3.2. Rats

When groups of 25 male and 25 female rats were fed 0.2, 0.5, 1.0 or 2.0% *trans*-anethole for 12 to 22 months, no effects on clinical chemistry, haematology, histopathology or mortality were reported. Dose levels were calculated (FDA, 1993) to be approximately 100, 250, 500 or 1000 mg/kg body weight/day. Delayed weight gain and decreased fat storage were recorded at the 500 and 1000 mg/kg body weight/day dose levels. In a paired feeding study, *trans*-anethole reduced the rate of weight gain (Le Bourhis, 1973b).

Anethole was administered to Sprague–Dawley CD rats in the diet for periods of 117–121 weeks. Six dose groups received the following concentrations of *trans*-anethole: 0% (52 male, 52 female; control 1); 0% (52 male, 52 female; control 2); 0.25% (78 male, 78 female); 0.5% (52 male, 52 female); 1.0% (52 male, 52 female); a stop-exposure group of 26 females and 26 males was added to assess the reversal of treatment related effects. The stop-exposure group received 1.0% *trans*-anethole in their diet for 54 weeks, after which 10 males and 10 females were randomly selected and maintained on the control diet until week 121. The remaining 16 males and 16 females in the stop-exposure group continued to receive the 1% *trans*-anethole diet. The author calculated the dose levels to be 100, 200 or 400 mg/kg body weight/day in males and 120, 250 or 550 mg/kg body weight/day in females for the low, intermediate and high dose groups, respectively (Truhaut *et al.*, 1989).

Parameters of the study included body weight, food and water consumption, daily clinical observations, and appearance of palpable masses from week 27 onwards. At necropsy, gross and histopathological examinations were conducted on the major organs and tissues of the animals. Also at the time of necropsy, haematological examinations were performed including erythrocyte count, haemoglobin concentration, mean corpuscular volume, haematocrit value, mean corpuscular haemoglobin concentration, leucocyte count and differential blood count.

No apparent reactions to treatment were noted during the course of the experiment. Adverse clinical signs were reported to occur only in animals that died or were killed when moribund and included decreased body weight, anorexia and lethargy. Most of the rats showed signs of sialodacryoadenitis, a viral disease. Mortality was slightly higher for females in the 1.0% dose group, but not for any other dose group. A decrease in body weight gain was reported for all treatment groups during the first 6 months of the experiment and was related to the poor palatability of the diet. After 6 months, the weight gain of the animals

administered 0.25% and 0.5% *trans*-anethole was similar to that of the control groups. The body weight gain of animals in the high dose group remained less than the animals in the control groups. In the stop-exposure group, the body weights of the animals were similar to those of the controls by the conclusion of the study.

The only treatment-related abnormality seen at necropsy was a reduction in adiposity in the highest dose group, principally in males. The authors attributed this to the decrease in food intake, not to a specific effect of the administration of *trans*-anethole. Haematological examination revealed minimal differences which were not related to the *trans*-anethole treatment.

Histological examination of the liver revealed the following non-neoplastic lesions which were considered treatment related by the authors: sinusoidal dilation (males at 1%; females at 0.5% and 1%); nodular hyperplasia (males at 0.5% and 1%; females at 1%); and hepatocytic hypertrophy (females at 0.5% and 1%) (see Table 3). Additionally, a slight increase in the incidence of hepatocellular carcinomas (controls 1/104; females at 1% 6/52) was reported in female rats at the highest dose level tested. The authors noted that the hepatocellular carcinomas were not diagnosed until week 98 and had no effect on the lifespan of the animal. Although the increase of hepatocellular carcinoma in female rats was determined to be statistically significant when compared to the other dose groups and controls, the incidence remained within the historical control range for the particular strain of rat. The authors reported a no-effect level of 0.25% for the dietary administration of *trans*-anethole to rats (Truhaut *et al.*, 1989), which corresponds to dose levels of 100 mg/kg body weight/day in males based on nodular hyperplasia at 200 mg/kg body weight/day and 120 mg/kg body weight/day in females based on sinusoidal dilatation and hepatocellular hypertrophy at 250 mg/kg body weight/day.

In consideration of new criteria developed for the evaluation of proliferative hepatic lesions in the rat, a group of pathologists formed a Pathology Working Group (PWG) to reassess the pathology data from the above study (Newberne *et al.*, 1989). The criteria used by the PWG were those followed by the National Toxicology Program (Maronpot and Boorman, 1982; Maronpot *et al.*, 1987). The PWG reported the following: (1) the frequency of the hepatic non-neoplastic changes seen in males and females fed the high dose (1.0%) and in females fed the mid-dose (0.5%) were characteristic of prolonged hepatic enzyme induction and chronic hepatic injury; (2) there was no difference in the incidence of neoplasms between the males of the control group and the males of any compound-treated group; (3) hepatocellular adenomas and carcinomas occurred late in the lifespan of high-dose female rats and generally developed in livers with numerous non-neoplastic lesions that were associated with cytotoxicity and tissue necrosis. Although the neoplastic lesions were statistically significant, clearly they were associated with hepatic injury. The latter observation is consistent with cytotoxicity observed upon chronic exposure *in vitro* to high hepatocellular concentrations of AE ($>10^{-4}$ mM) (Marshall and Caldwell, 1996).

The PWG concluded that *trans*-anethole does not represent a carcinogenic risk to humans (Newberne *et al.*, 1989). The dose levels of 100 mg *trans*-anethole/kg body weight/day in males and 120 mg *trans*-anethole/kg body weight/day in females that produced no hepatotoxic effects are more than 1000 times the estimated daily *per capita* intake ("eaters only") of 54 μg/kg body weight from use of *trans*-anethole as a flavouring substance.

2.2.4. Mutagenicity and genotoxicity studies

2.2.4.1. *In vitro*

In vitro studies of *trans*-anethole have shown no evidence of mutagenicity in *Salmonella typhimurium* (SAL) strains TA98, TA100, TA1535, TA1537 and TA1538 with and without metabolic activation with S9 at concentrations up to 25,000 mg/plate (Gorelick, 1995; Heck *et al.*, 1989; Mortelmans *et al.*, 1986; Nestmann *et al.*, 1980; Swanson *et al.*, 1979; To *et al.*, 1982) (see Tables 5 and 6). Anethole produced slight increases in the mutation frequencies in *Salmonella* strain T100 with S9 activation (Hsia, 1979; Sekizawa and Shibamoto, 1982). The increases observed at all doses tested were less than the two- or threefold increase generally considered necessary for a positive response. Other positive responses were limited to those SAL assays using non-standard protocols which included longer preincubation times and the use of excess S9 with and without cofactors (Marcus and Lichtenstein, 1982; Mortelmans *et al.*, 1986; Sekizawa and Shibamoto, 1982; Swanson *et al.*, 1979; To *et al.*, 1982). The results were not reproducible (Gorelick, 1995).

trans-Anethole exhibited no mutagenic potential in *Bacillus subtilis* in a rec assay or in *Escherichia coli* in a point mutation assay (Sekizawa and Shibamoto, 1982). *trans*-Anethole did not produce any evidence of mutagenicity in *Salmonella cerevisiae* (Nestmann and Lee, 1983). *trans*-Anethole (Gorelick, 1995; Heck *et al.*, 1989) induced an increase in mutations when incubated with L5178Ytk+/−mouse lymphoma cells (MLA), but only in the presence of S9 activation. The results of the MLA with activation for simple aliphatic and aromatic substances have been shown to be inconsistent with the results of other standardized genotoxicity assays (Heck *et al.*, 1989). The mouse lymphoma assay has poor selectivity for genotoxicity (Caldwell, 1993), due to its sensitivity to culture conditions of low pH and high osmolality (Scott *et al.*, 1991). *trans*-Anethole did not induce an

Table 5. Mutagenicity/genotoxicity studies for *trans*-Anethole *in vitro* results

Substance name	Test system *in vitro*	Test object	Concentration of substance	Results	Reference
trans-Anethole	Ames test	*S. typhimurium* TA98	not specified	Negative[20] Negative[21]	Marcus and Liechtenstein, 1982
trans-Anethole	Ames test	*S. typhimurium* TA100	not specified	Negative[20] Positive[21]	Marcus and Liechtenstein, 1982
trans-Anethole	Ames test	*S. typhimurium* TA98	3×10^3 μg/plate	Negative[20] Negative[21]	Swanson *et al.*, 1979
trans-Anethole	Ames test	*S. typhimurium* TA100	3×10^3 μg/plate	Positive [20] Positive[21]	Swanson *et al.*, 1979
trans-Anethole	Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	7 to 7×10^3 μg/plate	Negative[23] Negative[24] Positive[25]	To *et al.*, 1982
trans-Anethole	Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	60 to 600 μg/plate	Negative[20] Positive[21]	Sekizawa and Shibamoto, 1982
anethole (isomer not specified)	Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0, 2, 20, 200 mg/plate	Positive[26,27]	Hsia *et al.*, 1979
trans-Anethole	Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0 to 200 μg/plate	Negative[28]	Nestmann *et al.*, 1980
trans-Anethole	Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0 to 2.5×10^4 μg/plate	Negative[25]	Heck *et al.*, 1989
trans-Anethole	Ames test	*S. typhimurium* TA98, TA100, TA1535, TA1537	0 to 280 μg/plate	Negative[25]	Mortelmans *et al.*, 1986
trans-Anethole	Ames test	*S. typhimurium* TA100	25 to 500 μg/plate	Negative[23,29]	Gorelick, 1995
trans-Anethole	Ames test	*S. typhimurium* TA100	100 to 750 μg/plate	Negative[23,30]	Gorelick, 1995
trans-Anethole	DNA repair test (Rec assay)	*B. subtilis* strains H17 Rec^+ and M45 Rec^-	1×10^4 μg/disk	Negative[31]	Sekizawa and Shibamoto, 1982
trans-Anethole	Mutagenicity assay	*S. cerevisiae* strains D7 and xv185-14C	Not specified	Negative[31]	Nestmann and Lee, 1983
trans-Anethole	Mutagenicity assay (uvrA reversion test)	*E. coli* strain WP2 trp-	60 to 600 μg/plate	Negative[31]	Sekizawa and Shibamoto, 1982
trans-Anethole	Mouse lymphoma assay (TK ± cell)	Mouse lymphoma L5178Y cells	15.6 to 31.3 μg/ml	Negative[20] Positive[21]	Heck *et al.*, 1989
trans-Anethole	Mouse lymphoma assay (TK ± cell)	Mouse lymphoma L5178Y cells	48 to 84 μg/ml without S9; 20 to 72 μg/ml with S9	Negative[20] Positive[21]	Gorelick, 1995
trans-Anethole	Chromosomal aberration	Chinese hamster ovary cells	0.025 to 0.2 μg/ml without S9; 0.013 to 0.1 μg/ml with S9	Negative[20] Positive[21]	Gorelick, 1995
trans-Anethole	DNA repair test (UDS)	Rat hepatocytes (primary culture)	0.15 μg/ml to 1.5×10^3 μg/ml	Negative	Howes *et al.*, 1990b
trans-Anethole	DNA repair test (UDS)	Rat hepatocytes (primary culture)	0 to 30 μg/ml	Negative	Heck *et al.*, 1989
trans-Anethole	DNA repair test (UDS)	Rat hepatocytes (primary culture)	0.15 μg/ml to 1.5×10^3 μg/ml	Negative	Caldwell *et al.*, 1992
trans-Anethole	DNA repair test (UDS)	Rat hepatocytes (primary culture)	0.15 μg/ml to 1.5×10^3 μg/ml	Negative	Marshall and Caldwell, 1992, 1996
trans-Anethole	DNA repair test (UDS)	Rat hepatocytes (primary culture)	1.5 μg ml to 1.5×10^3 μg/ml	Positive[32]	Muller *et al.*, 1994
anethole 1,2-epoxide	DNA repair test (UDS)	Rat hepatocytes (primary culture)	0.15 μg/ml to 1.5×10^3 μg/ml	Negative	Marshall and Caldwell, 1996
anethole 1,2-diol	DNA repair test (UDS)	Rat hepatocytes (primary culture)	0.15 μg/ml to 1.5×10^3 μg/ml	Negative	Marshall and Caldwell, 1996
anethole 1,2-oxide	DNA repair test (UDS)	Rat hepatocytes (primary culture)	not specified	Negative	Caldwell *et al.*, 1992
3′-hydroxyanethole	DNA repair test (UDS)	Rat hepatocytes (primary culture)	not specified	Negative	Caldwell *et al.*, 1992

[20]Without S13 activation. [21]With S13 activation. [22]With S13 and NADPH. [23]Without S9 activation. [24]With S9 activation. [25]With S9 + cofactor PAPS (3′-phosphadenosine-5′-phosphosulfate). [26]With S9 or S13 activation. [27]Weakly positive in TA100 but not a dose-related response. [28]With and without S9 and S13 activation. [29]Tested with 40% S9 and NADPH generating system. [30]Tested with 10% S9 and PAPS. [31]Tested without any metabolic activation system. [32]Slight increase in UDS.

Table 6. Mutagenicity/genotoxicity studies for alkoxypropenylbenzene derivatives *in vivo* results

Substance name	Test system *in vivo*	Test object	Dose level	Results	Reference
Anethole	DNA post-labelling assay	mouse	0.7 mg/mouse	Negative[33]	Randerath *et al.*, 1984
Anethole	DNA post-labelling assay	mouse	2 to 10 mg/mouse	Negative	Phillips *et al.*, 1984
trans-Anethole	micronucleus assay	mouse	2.5 mg/kg body weight	Negative	Abraham, 1996
trans-Anethole	micronucleus assay	mouse	2 ml/kg body weight	Negative	Siou *et al.*, 1984
Anethole	micronucleus assay	mouse	250, 500 or 1000 mg/kg body weight/day for 7 days	Negative	Al-Harbie *et al.*, 1995
trans-Anethole	micronucleus assay	mouse	0.25 or 0.5 g/kg body weight	Negative	Marzin, 1979
trans-Anethole	DNA repair test (UDS)	rat hepatocytes	0 to 500 mg/kg body weight	Negative	Marshall and Caldwell, 1996

[33]The rodent carcinogens safrole and estragole produced 150 and 220 times the number of adducts when compared with number of adducts formed following the administration of anethole (Marshall and Caldwell, 1996).

increase in chromosomal aberrations with and without S9 activation (Gorelick *et al.*, 1995).

Concentrations of 10^{-2} to 10^{-6} M *trans*-anethole, AE or anethole-1,2-diol failed to give any evidence of unscheduled DNA synthesis when incubated with hepatocytes isolated from female SD-CD rats (Caldwell *et al.*, 1992; Howes *et al.*, 1990a,b; Marshall and Caldwell, 1992, 1996; Muller *et al.*, 1994).

Considering the results of standard SAL assays and mutagenicity tests in other bacterial strains and the results of unscheduled DNA assay with anethole or its metabolites, the weight of evidence suggests that *trans*-anethole is not genotoxic. The few reports of mutagenicity of anethole in assays using non-standard protocols were not reproducible and are not supported by consistent negative results in standard SAL assays for anethole.

2.2.4.2. *In vivo*

In vivo mammalian tests with *trans*-anethole have produced negative results. In three separate mouse micronucleus assays, *trans*-anethole did not induce SCE or chromosome aberrations in $B6C3F_1$ mouse bone marrow cells following ip injections of doses ranging from 2.5 to 2000 mg/kg body weight (Abraham, 1996; Marzin, 1979; Siou *et al.*, 1984). Groups of Swiss albino mice were adminstered 250, 500 or 1000 mg anethole/kg body weight/day for 7 days. Examination of the femoral bone marrow cells revealed no increase in the number of micronucleated cells. The ratio of polychromatic to normochromatic erythrocytes was altered indicating mitodepression (i.e. cytotoxicity) (Ali-Harbi *et al.*, 1995).

In an *in vivo/in vitro* assay, SD-CD female rats were given a single oral dose of 0, 1, 125 or 500 mg *trans*-anethole/kg body weight in corn oil by gavage. Hepatocytes isolated 16 hours after administration failed to exhibit any evidence of unscheduled DNA synthesis (Marshall and Caldwell, 1996). Compared with safrole and estragole, anethole failed to induce a significant increase in DNA adducts in mouse bone marrow cell when mice were given a single ip injection of 0.7 to 10 mg (Phillips *et al.*, 1984; Randerath *et al.*, 1984).

The consistent negative responses obtained *in vitro* and *in vivo* genotoxicity studies, including the results of the rat *in vivo/in vitro* UDS and *in vitro* UDS with rat hepatocytes, lead the Panel to conclude that *trans*-anethole and AE are not genotoxic. These data support the conclusion that the hepatocarcinogenic effects observed in female rats in the 2+-year study of *trans*-anethole occur via a non-genotoxic mechanism in which carcinogenicity is secondary to dose-dependent chronic cytotoxicity.

2.2.5. Reproduction/developmental studies

In a four-generation reproduction study, groups of 40 male and 40 female Wistar rats were administered *trans*-anethole in the diet at a concentration

of 1% from weaning to 3 months of age. After dosing, control males were mated with control and treated females; control females were mated with treated males; and treated males were mated with treated females. The treated male/treated female group continued to be dosed during the mating period. Following 70 days of treatment, the offspring of the control male/control female and treated male/treated female rats were mated to produce a second generation. Third and fourth generations were also produced in this manner. The only treatment-related effect was a decrease in the weight of pups surviving to 2 weeks from dams fed the diet containing 1% *trans*-anethole (approx. 700 mg/kg body weight/day) and a delay in body weight gain compared with pups delivered from control dams.

In a second experiment, rats born from control parents were paired with rats from treated parents. Pups from treated parents were fed by control dams while pups from control parents were fed by treated dams. Delayed growth was reported for both groups of pups. These data indicated that growth was affected only by the dam's diet. The author concluded that the only effect of *trans*-anethole was a delay in the growth of pups resulting from the reduced palatability of the *trans*-anethole containing diet (Le Bourhis, 1973a).

Groups of 10 female Crl:CD BR rats were administered *trans*-anethole via gavage at doses of 0, 25, 175 or 350 mg/kg body weight/day for 7 days prior to mating and until scheduled termination on day 4 of lactation. Statistically significant reductions in weight gain were reported in the 175 and 350 mg/kg body weight/day dose groups during the 7-day premating period. During the gestation period and lactation period, statistically significant reductions in average weight gain and a reduction in average food consumption values were reported in the high-dose animals. Compared with the control groups, litters of dams treated at the 350 mg/kg body weight/day revealed slight increases in gestation time, increases in pup mortality and stillbirths and reductions in body weight at birth and at the conclusion of the study. No gross physical anomalies of the pups were attributed to treatment with *trans*-anethole. *trans*-Anethole did not produce any reproductive toxicity at doses which are not associated with palatability problems (ARL, 1992).

3.0. Exposure

3.1. Flavour use

trans-Anethole is used as a flavouring substance in foods at approximate levels ranging from 2.5 ppm in gravies to 1500 ppm in chewing gum. Total annual volume from use of *trans*-anethole as a flavouring substance in the USA is 17,100 kg (NAS, 1987). Based on the most recent annual volume reported in the USA (NAS, 1987), the estimated daily *per capita* intake ("eaters only") of *trans*-anethole from use as a flavouring substance is 54 μg/kg body weight/day.

3.2. Natural occurrence

trans-Anethole has been detected in a wide variety of foods (see Table 4) and is a constituent of the volatile component of more than 20 spices. Its concentration in sweet (var. *dulce*) and bitter fennel (var. *vulgare*) and anise may account for as much as 90% of the volatile component. It occurs at lower concentrations in the volatile component of lemon balm (6.1%), coriander (0.5%) and sweet basil (0.08%) and at very low concentration in apples (< 2 ppm) (CIVO–TNO, 1994). Dried fennel seeds contain up to 6% volatile oil of which approximately 50–60% is *trans*-anethole. Anise seed contains up to 4% oil of which 80–90% is *trans*-anethole (Farrell, 1985). Based on the reported annual volumes of 443,000 kg and 70,500 kg (NAS, 1987) of fennel seed (*Foeniculum vulgare M.* and var. *dulce)* and anise seed (*Pimpinella anisum* L.), respectively, the annual intake of *trans*-anethole as a component of these two spices is estimated to be 15,546 kg, which is approximately equal to its use as a flavouring substance (17,100 kg). The estimated annual intake of *trans*-anethole from use as a flavouring substance *and* as a component of fennel seed and anise seed is approximately 30,000 kg in the USA.

4.0. Discussion

As measured by oral LD_{50} values, *trans*-anethole is acutely toxic to mice and rats at dose levels of more than 1800 mg/kg body weight and more than 2000 mg/kg body weight, respectively (see Table 2). In a 90-day dietary study, *trans*-anethole showed no evidence of toxicity in mice at dose levels 240 mg/kg or more body weight/day (Minnema, 1997). Toxicity could not be studied at higher dietary levels because both male and female mice suffered the effects of "inanition syndrome" associated with an unpalatable diet. Minimal enzyme changes, decreased hepatocyte glycogen content, and an increased incidence of hepatocyte hypertrophy observed at dose levels 240 mg/kg or more body weight/day were concluded (Minnema, 1997; Newberne, 1997) to be responses to a poor diet and an increased need to metabolize *trans*-anethole. As such, they were considered adaptive physiologic responses and not pathologic alterations.

In a 90-day dietary study in rats, *trans*-anethole was reported to be hepatotoxic to both sexes of Sprague–Dawley rats at target dose levels 600 or more based on minimal to slight single cell necrosis associated with perivascular inflammatory infiltrate in males and increased γ-glutamyltransferase values in females (Minnema, 1997). An independent evalu-

ation of the liver histology (Newberne, 1997) concluded that the incidence and grade of hepatocellular necrosis in test animals was similar to that for controls. An increased incidence of hepatocyte hypertrophy observed at 300 mg/kg or more body weight/day in males and at 600 mg/kg or more body weight/day in females was concluded to be the result of enzyme induction associated with an increased requirement for *trans*-anethole metabolism. The NOAEL in the 90-day rat study was concluded to be at 300 mg/kg body weight/day (Minnema, 1997).

In the 2+-year study, Sprague–Dawley rats exhibited evidence of hepatotoxicity at dietary levels of 200 and 400 mg/kg body weight/day for males and 250 and 550 mg/kg body weight/day for females based on a statistically increased incidence of focal and nodular hyperplasia, sinusoidal dilatation, and distended bile ducts (Truhaut *et al.*, 1989). An independent histopathological evaluation confirmed the presence of hepatic injury at these dose levels (Newberne *et al.*, 1989). Additionally, a statistically significant increase in hepatocellular carcinoma was reported in female rats at the highest dose levels (i.e. 550 mg/kg body weight/day) (Newberne *et al.*, 1989; Truhaut *et al.*, 1989). The neoplasms occurred in livers with significant non-neoplastic lesions indicative of hepatotoxicity and necrosis (Newberne *et al.*, 1989). Based on the evidence of hepatotoxicity in males at 200 mg/kg or more body weight and females at 250 mg/kg or more body weight, the NOAEL for the 2+-year study was concluded to be 100 mg/kg body weight/day for males and 120 mg/kg/body weight/day for females (Newberne *et al.*, 1989; Truhaut *et al.*, 1989).

The biochemical and toxicity data on *trans*-anethole strongly suggest that the hepatotoxicity observed in rats exposed to *trans*-anethole is associated with the dose-dependent metabolic formation of anethole epoxide (AE). At high dose levels (100 mg or more *trans*-anethole/kg body weight), a metabolic shift to greater epoxidation in rats leads to increased hepatocellular concentrations of AE. Epoxidation is more pronounced in rats than mice (see Section 2.1.1.). Therefore, the rat is considered to be the most sensitive species for evaluating the potential for AE-related hepatotoxicity in humans exposed to *trans*-anethole from use as a flavouring substance.

At low doses of *trans*-anethole, AE is readily detoxicated by enzymes such as epoxide hydrolase (EH) and glutathione *S*-transferase (GST). With increasing dose levels of *trans*-anethole, hepatic levels of AE increase and these enzymes (especially EH) approach saturation leading to cytotoxicity. Inhibition of EH and GST has been associated with an increase in the cytotoxic effects of *trans*-anethole (see Section 2.1.1.4.3.). AE is approximately 10 times more cytotoxic than *trans*-anethole in the hepatocytes of rats. This difference corresponds approximately to the proportion (12–18%) of *trans*-anethole that is metabolized to AE in rats at dose levels (200 mg or more *trans*-anethole/kg body weight/day) required to observe hepatotoxicity. Taken together, these data suggest that cytotoxicity and heptatoxicity are linked metabolically to the formation of AE in the liver.

The effect of sex and dose on the daily production of AE in rats is presented in Fig. 1. Clearly, daily production of AE is significantly higher in female rats as compared with males. The difference reflects increased epoxidation in females compared to males at higher dose levels. At dose levels (i.e. 200 to 550 mg/kg body weight/day) of *trans*-anethole which resulted in hepatotoxic effects in male and female rats for 2+ years, daily hepatic production of AE levels was in the range of 30 to 120 mg/kg body weight (see Section 2.2.3.2. and Fig. 1). The NOAEL of 120 mg *trans*-anethole/kg body weight/day for female rats in the 2+-year study corresponds to production of approximately 22 mg AE/kg body weight/day*, which is more than 10,000 times the level of 0.002 mg AE/kg body weight/day† produced by humans from intake of *trans*-anethole as a flavouring substance.

Daily exposure to significant levels of AE must continue over a long duration in order to observe the onset of hepatotoxicity in rats. No significant hepatotoxicity was observed in male or female rats at dose levels up to 300 mg *trans*-anethole/kg body weight/day for 90 days (Minnema, 1997), which correspond to daily AE levels up to 55–60 mg/kg body weight/day‡. While these levels of AE are within the range (30–120 mg AE/kg body weight/day) shown to induce hepatotoxicity in the 2+-year study, they did not produce adverse hepatic effects after 90 days. Taken together, these data suggest that both AE concentration and duration of exposure to AE are critical to the onset of hepatotoxicity. It is postulated that continuous exposure to high dose levels of *trans*-anethole leads to a continuum of biochemical and toxicological events: (1) saturation of detoxication pathways; (2) concomitant increase in hepatocellular concentrations of AE; (3) cytotoxicity; (4) cell death (necrosis); (5) hepatocyte proliferation, and ultimately (6) liver tumours in a few female rats.

The low incidence (6/52) of carcinomas were observed in the severely compromised livers of female rats administered 550 mg *trans*-anethole/kg body weight/day for 2+-years. The fact that hepatocellular carcinomas occurred only in the female

*Calculated as x = (120 mg *trans*-anethole/kg body weight) [18% epoxidation (Bounds, 1994)].

†Calculated as x = (0.054 mg *trans*-anethole/kg body weight/day) [3% epoxidation (Sangster *et al.*, 1987)].

‡Calculated as x = (300 mg *trans*-anethole/kg body weight) [18–23% epoxidation (Bounds, 1994)].

rat is a reflection of a higher daily dose of *trans*-anethole, increased conversion to AE compared with the male, and decreased detoxication of AE by the female which exhibits a lower activity of the AE-detoxication enzyme EH compared with that of the male (Meijer *et al.*, 1987).

The available mutagenicity and genotoxicity data demonstrate that neither anethole nor AE are genotoxic (see Section 2.2.4.). The pattern of significant induction of Phase II conjugation enzymes (GT and GST) and the weak induction of Phase I CYP450 monooxygenase enzymes by *trans*-anethole, along with the incidence of hepatocyte hypertrophy (see Section 2.2.2.) and increased relative liver weights in mice and rats (Reed and Caldwell, 1992b; Rompelberg *et al.*, 1993) are phenomena associated with an increased requirement for metabolism of *trans*-anethole and are widely observed with other non-genotoxic hepatotoxic substances (Grasso and Hinton, 1991). Therefore, the weight of evidence indicates that the neoplasms observed in the liver of female rats at a dose level of 550 mg *trans*-anethole/kg body weight/day occurred secondary to dose-dependent hepatotoxicity resulting from continuous exposure to high hepatocellular concentrations of anethole epoxide.

The conclusion that *trans*-anethole-induced carcinogenicity in female rats is secondary to hepatotoxicity is significant to the overall safety evaluation of *trans*-anethole from use as a flavouring substance because hepatotoxicity is a threshold phenomenon. It has recently been proposed that a default assumption of non-linearity is appropriate when carcinogenicity results from a secondary effect of toxicity (*Federal Register* **61** (79), 17981, 23 April 1996). In the 2+-year study in female rats, hepatotoxicity was reported when dietary levels of *trans*-anethole were 250 mg/kg or more body weight/day and hepatic production of AE was 40 mg/kg more than body weight/day*. However, hepatocellular neoplasms were reported in severely compromised livers of female rats only when dietary levels increased to 550 mg *trans*-anethole/kg body weight/day and hepatic production of AE was 120 mg/kg more than body weight/day†. These observations strongly suggest that *trans*-anethole-induced carcinogenicity in female rats is a threshold effect.

5.0. Conclusion

In 1993, the Panel initiated a comprehensive program to re-evaluate the status of all FEMA GRAS flavouring substances concurrent with a systematic revision of the FEMA Scientific Literature Reviews (SLRs). Scientific data related to the safety of *trans*-anethole were evaluated in this monograph. *trans*-Anethole was reaffirmed as GRAS (GRASr) in 1997 based on the recognized metabolic detoxication of *trans*-anethole in humans at low levels of exposure (1 mg/kg body weight/day); its low level of use as a flavouring substance (54 μg/kg body weight/day); the safety factors calculated from results of subchronic and chronic studies (> 1000 to > 10,000); the lack of genotoxicity and mutagenicity observed for *trans*-anethole and AE; and the conclusion that the statistically significant increase in the incidence of hepatocellular carcinomas in the high dose group of female rats fed *trans*-anethole for 2+ years was secondary to pronounced hepatotoxicity associated with continuous high hepatocellular levels (> 120 mg/kg body weight/day) of AE. Under conditions of intended use as a flavouring substance, *trans*-anethole does not pose a risk to human health. The safety of *trans*-anethole is supported by its occurrence as a natural component of traditional foods.

6.0. References

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*Calculated as x = (250 mg *trans*-anethole/kg body weight/day) [18% epoxidation (Bounds, 1994)].

†Calculated as x = (550 mg *trans*-anethole/kg body weight/day) [23% epoxidation (Bounds, 1994)].

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