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**The Flavor and Fragrance High Production Volume
Consortia**

The Terpene Consortium

Test Plan for Estragole

Estragole

CAS No. 140-67-0

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FFHPVC Terpene Consortium Registration Number

**Submitted to the EPA under the HPV Challenge Program by:
The Flavor and Fragrance High Production Volume Chemical Consortia
1620 I Street, NW, Suite 925
Washington, DC 20006
Phone: 202-331-2325
Fax: 202-463-8998**

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1.1.1 Summary of Key Hazard Data for Estragole

ENDPOINT	SUBSTANCE/SURROGATE/ CHEMICAL CATEGORY ¹	VALUE/RANGE ²	REFERENCE
Physical Properties			
<i>Vapor pressure</i>	Estragole (4-methoxyallylbenzene)	0.041 mm Hg (21°C) 1.0 mm Hg (52.6 °C)	Daubert, 1989 Stull, 1974
<i>Partition Coefficient</i>	Estragole (4-methoxyallylbenzene)	3.47	MackKay, 2000
Environmental Fate			
<i>Biodegradation</i> ³	4-Hydroxy-3-methoxyallylbenzene)	+ (OECD 301B)	Quest, 1994
Ecotoxicity			
<i>Fish</i>	3,4-Dimethoxyallyl benzene(methyl eugenol)	96-hr LC50=8.1 mg/L (bluegill) 96-hr LC50=6.0 mg/L (trout)	Beroza, 1975
<i>Aquatic Invertebrates</i>	Estragole (4-methoxyallylbenzene)	48-hr EC50=8.7 mg/L (Daphnia magna) 48-hr LC50=6.0 mg/L NOEC=4.73 mg/L	Ward, 2003
<i>Aquatic Plant</i>	Estragole (4-methoxyallylbenzene)	72-hr EC50= 1.35 mg/L using the number of cells/mL. The 72-hr NOEC=0.118 mg/L	Boeri, 2003,
Human Health			
<i>Repeat Dose</i> ⁴ (route)	Estragole (4-methoxyallylbenzene)	NOEL=50 mg/kg bw/d (rat, dietary) NOAEL=75 mg/kg bw/d (mouse, gavage) LOAEL=150 mg/kg bw/d (mouse, gavage) LOAEL=37.5 mg/kg bw/d (rat, gavage)	Jones, 2003 NTP, 2005
<i>Reproduction (route)</i>	3,4-Dimethylene oxyallylbenzene (safrole)	NOAEL(maternal toxicity)=5 mg/kg bw/d (mouse, gavage) LOAEL(maternal toxicity)=50 mg/kg bw/d (mouse, gavage) NOAEL(fetal toxicity)=5 mg/kg bw/d (mouse, gavage) LOAEL(fetal toxicity)=50 mg/kg bw/d (mouse, gavage)	Moro, 1985
	3,4-Dimethoxyallyl benzene (methyl eugenol)	NOAEL for effects to reproductive organs (epididymus/seminal vesicles/tunica vaginalis/scrotal sac/prostrate/testes or ovaries)= 300 mg/kg bw/d (m,f rats)	NTP, 2002

¹ Surrogate is a structurally related substance that may include a metabolic product or precursor of the named substance. Range of values may be reported for substance, surrogate or chemical category.

² Experimental value or values for a substance or group of substances in the chemical category

³ not biodegradable, (-); readily biodegradable, (+); ready and ultimately biodegradable, (++)

⁴ Value is the NOAEL or NOEL(route, duration)

Developmental (route)	3,4-Dimethylenoxyallylb enzene	LOAEL(maternal toxicity)=50 mg/kg bw/d (mouse, gavage) NOAEL(teratogenic effects)=200mg/kg bw/d (mouse, gavage) LOAEL(fetal toxicity)=50 mg/kg bw/d (mouse, gavage)	Moro, 1985
	Nutmeg oil (contains 80% terpene hydrocarbons –see developmental effects for monoterpene and bicyclic terpene hydrocarbons;20% of allylalkoxybenzene derivatives	NOEAL(teratogenic effects)= 52 mg/kg bw/d as allylalkoxybenzene derivatives) (rat, f, gavage)	Morgeiridge, 1973c
Genotoxicity⁵			
In vitro	Estragole (4- methoxyallylbenzene)	+ UDS + Rec	Howes, 1990 Chan, 1992 Muller, 1994 Zani, 1991
In vivo	Estragole (4- methoxyallylbenzene)	+ DNA adducts P ³² -post labeling + DNA adducts P ³² -post labeling	Randerath, 1984 Phillips, 1981

⁵ (-), no significant genotoxic potential; (=/-), equivocal evidence; (+), positive evidence of genotoxicity. AMS, Ames assay; MLA, Mouse Lymphoma assay; ABS, chromosomal aberration assay; UDS, Unscheduled DNA Synthesis; MN, Micronucleus test, SCE, Sister Chromatid Exchange assay, SLA, Sex-linked Lethal assay.

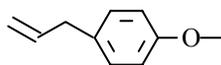
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The Flavor and Fragrance High Production Volume Consortia

Test Plan for Estragole

2 IDENTITY OF SUBSTANCE



Estragole

CAS No. 140-67-0

Synonyms:

p-Allylanisole
Benzene, 1-methoxy-4-(2-propenyl)-
Chavicol methyl ether
Isoanethole
p-Methoxyallylbenzene
1-Methoxy-4-(2-propen-1-yl)benzene

3 CATEGORY ANALYSIS

3.1 INTRODUCTION

In October of 1999, members of the U.S. flavor and fragrance industries as well as other manufacturers that produce source materials used in flavors and fragrances formed consortia of companies in order to participate in the Chemical Right-to-Know Program. Members of these consortia are committed to assuring the human and environmental safety of substances used in flavor and fragrance products. The consortia are organized as the Flavor and Fragrance High Production Volume Consortia (FFHPVC). The terpene consortium, as a member of FFHPVC, serves as an industry consortium to coordinate testing activities for terpene substances under the Chemical Right-to-Know Program. Twenty-one (21) companies are current members of the Terpene Consortium. The Terpene Consortium and its member companies are committed to assembling and reviewing available test data, developing and providing test plans for each of the sponsored chemicals, and where needed, conducting additional testing. The test plan, category analysis and robust summaries presented represent the first phase of the Consortium's commitment to the Chemical Right-to-Know Program.

3.2 BACKGROUND INFORMATION

This category analysis and test plan provides data for estragole. Estragole is currently permitted by the U.S. Food and Drug Administration (FDA) for direct addition to food for human consumption as a flavoring substance and is considered by the Flavor and Extract Manufacturers' Association (FEMA) Expert Panel to be "generally recognized as safe" (GRAS) for its intended use as a flavoring substance [Hall and Oser, 1965]. Estragole occurs naturally in more than 39 foods [CIVO-TNO, 2000]. Exposure to estragole occurs principally through consumption of spices such as tarragon and essential oils derived from spices. Estragole is also added directly to food as a flavouring substance. Estragole modifies spice flavors and seasonings for condiments and meats. It

is also used in heavy fruit, root beer, and anise-type flavors. The estimated poundage of estragole added directly as a flavoring substance was reported to be approximately 500 kg [Lucas *et al.*, 1999].

Major sources of oral exposure occur *via* intake of basil, tarragon, anise, and bitter fennel. Greater than 90% of the mean daily *per capita* intake (1.0 micrograms/kg bw per day) of estragole is derived from consumption of tarragon, basil, fennel, anise and their essential oils. Based on the conservative assumption that only 10% of the U.S. population consumed foods containing estragole, the estimated daily *per capita* intake (“eaters only”) of estragole from all sources is less than 10 micrograms/kg bw per day.

3.3 STRUCTURAL CLASSIFICATION

Estragole is 4-methoxyallylbenzene. Estragole is a C₁₀ terpene that is recognized chemically as 4-methoxyallylbenzene. In plants, it is formed via the shikimic acid pathway. As a terpene derivative it is closely related in structure to other naturally occurring plant constituents containing a 4-alkoxyallylbenzene nucleus. Methyl eugenol (3,4-dimethoxyallylbenzene), elemicin (3,4,5-trimethoxyallylbenzene), myristicin (3-methoxy-4,5-methylenedioxyallylbenzene), and safrole (4,5-methylenedioxyallylbenzene) are all examples of *p*-alkoxyallylbenzene derivatives that can be found in spices such as nutmeg and basil. The only structural difference between estragole and these other alkoxyallylbenzene derivatives is the presence of additional ring alkoxy substituents (*i.e.*, methyl eugenol has a second ring methoxy group). *p*-Alkoxyallylbenzene derivatives participate in the same primary pathways of absorption, metabolism and excretion and exhibit the similar toxicologic endpoints (*i.e.* liver). Therefore, key data on *p*-alkoxyallylbenzene derivatives provide a more comprehensive chemical, biological and toxicological characterization of estragole.

Although structurally related to estragole and metabolically similar at lower levels of exposure, anethole does not participate in the well recognized intoxication pathway

utilized by estragole. The structures of estragole and anethole (CAS No. 104-46-1) differ only in the position of the side-chain double bond. Estragole is 4-(2-propenyl)anisole while anethole is 4-(1-propenyl)anisole. Their similar physical properties reflect the small difference in chemical structure. The presence of an allyl side chain versus a 1-propenyl side chain has an impact on the animal metabolism of each substance at high levels of exposure. Both substances are primarily detoxicated *via* *O*-demethylation at low levels of exposure (see below and the Test Plan for Anethole). At higher intake levels (greater than 50 to 100 mg/kg bw), estragole participates, to a significant extent, in a metabolic pathway (1'-hydroxylation) that, upon repeated daily exposure, is associated with hepatic toxicity. At these higher levels of intake, anethole mainly participates in a detoxication pathway (oxidative cleavage to yield a benzoic acid derivative) (see below). Therefore, human health toxicity data on anethole are considered relevant to estragole at low levels of exposure at which both substances participate in common pathways of metabolic detoxication (*e.g.*, *O*-demethylation) (see section 2.5 below).

3.4 INDUSTRIAL AND BIOGENIC PRODUCTION

The vast majority of estragole used as a flavoring agent in food is isolated from exotic (Reunion-type) basil that can contain as much as 90% estragole in the essential oil. Production of estragole from this source and other essential oils is approximately 10 metric tons annually [Bauer and Garbe, 1985]. However, the vast majority of estragole isolated from nature is as a component of crude sulfate turpentine (CST). Fractions containing estragole, anethole, and caryophyllene account for 1-2% of commonly distilled CST [Derefer and Traynor, 1992]. Although this represents only a small portion of CST, the sheer volume of production of CST on an annual basis provides the majority of estragole used for commercial purposes in food flavors, fragrances, cosmetics, and household products. Crude sulfate turpentine is fractionated into an anethole/caryophyllene mixture (0.5-1%) and an azeotropic mixture of estragole and *alpha*-terpineol (1%). The majority of estragole present in this mixture is catalytically isomerized to anethole by the action of potassium hydroxide. The resulting mixture of anethole (mainly *trans*-anethole) and *alpha*-terpineol is further separated by fractional

crystallization [Bauer and Garbe, 1985]. The majority of estragole isolated from CST, is converted to *trans*-anethole.

In 1977, it was reported that the annual production of CST in the United States was 92,750 tons (185,500,000 pounds). Based on the annual volume of production of CST and the estragole content in CST (1%), it can be estimated that the potential amount of estragole isolated from CST is 1,855,000 pounds or 843,000 kg (843 metric tons).

Level III fugacity calculations indicate that, in the environment, estragole partitions mainly to the soil and water with less than 1% passing into the atmosphere. In the atmosphere, the relatively small amount of estragole rapidly reacts (half-life equals 3.9 hours) with hydroxyl radicals, ozone and nitrate radicals [Mackay, 1996a, 1996b]. Of more than 50 volatile organic compounds emitted by vegetation into the atmosphere, estragole was classified as exhibiting a relatively high rate of reactivity with hydroxyl radicals [Atkinson, 1990]. If it were conservatively assumed that 2% of industrially separated estragole is lost during industrial processing of CST, the vast amount (16.8 metric tons) would partition to the soil and water while the total annual estragole emitted into the atmospheric emission would be insignificant (0.17 metric tons). Compared to the amount (10 metric tons) consumed as a constituent of a traditional diet, exposure to industrial estragole loss to the atmosphere is insignificant. As a plant terpene, estragole is a normal component of the earth's atmosphere [Guenther *et al.*, 1995]. However, in the absence of quantitative data on the emission rates of estragole from vegetation, it is not currently possible to estimate its annual rate biogenic production.

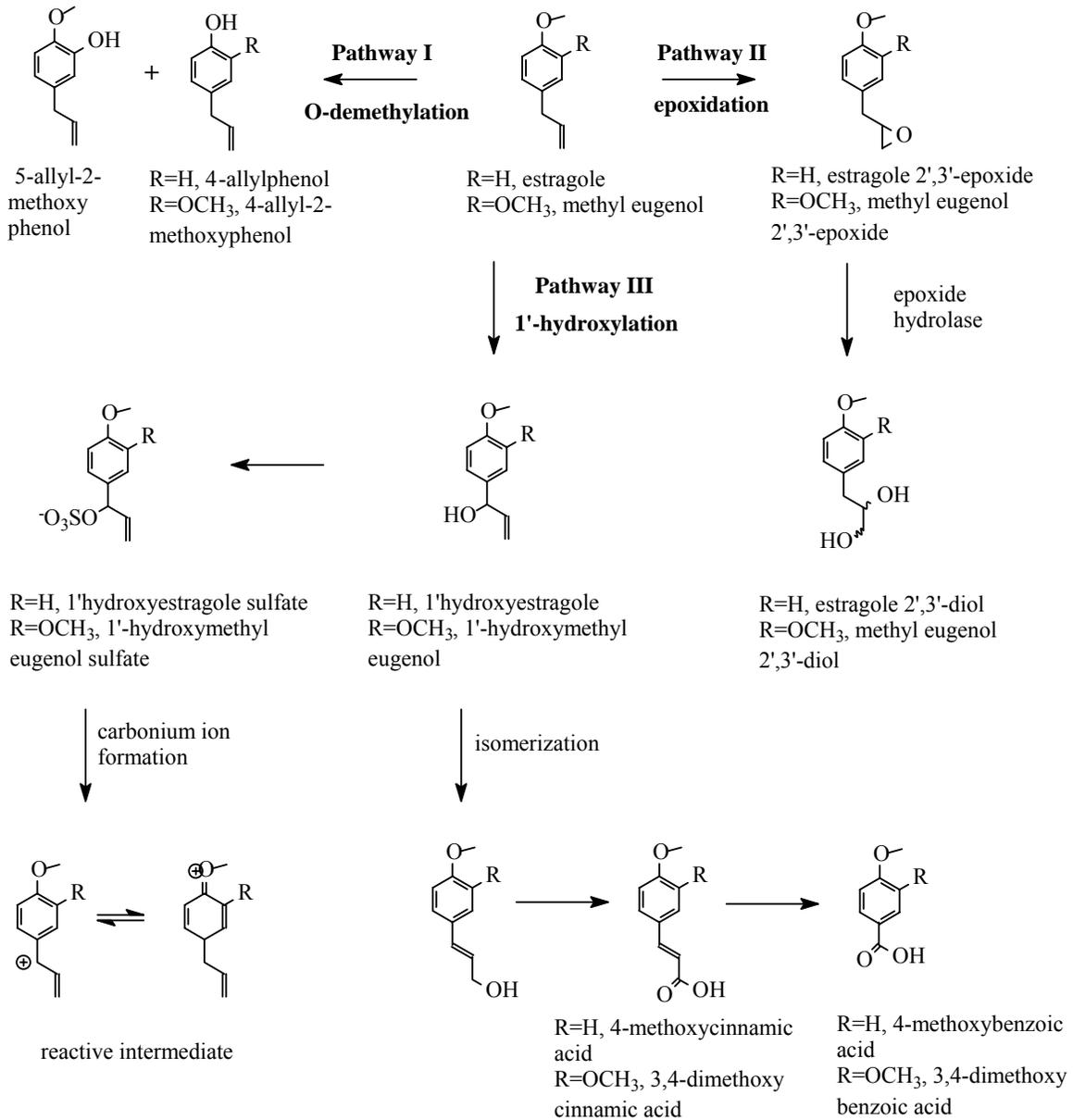
3.5 CHEMICAL REACTIVITY AND METABOLISM

The pharmacokinetic and metabolic pathways of estragole and methyl eugenol have been extensively reviewed in a recent publication (see Figure 1) [Smith *et al.*, 2002]. Estragole undergoes rapid and essentially complete absorption *via* the oral route [Anthony *et al.*, 1987; Sutton *et al.*, 1985]. The metabolism of estragole and structurally related substances (*i.e.* methyl eugenol and *trans*-anethole) is clearly dose dependent. At low

dose, (less than 10 mg/kg bw) estragole is primarily *O*-demethylated to yield the corresponding phenol derivative that is conjugated with glucuronic acid or sulfate and excreted mainly in the urine. Minor metabolic options at low levels of exposure include epoxidation of allyl double bond or 1'-hydroxylation at the benzylic position of the allyl side chain. As the dose is increased (0.05 to 1000 mg/kg bw) in mice and rats, the *O*-demethylation pathway 1'-hydroxylation becomes saturated [Zangouras *et al.*, 1981; Sangster *et al.*, 1987]. The 1'-hydroxylation pathway has been shown to be a significant metabolic activation pathway leading to hepatotoxic effects in mice and rats [Miller *et al.*, 1983; Phillips *et al.* 1981; Swanson *et al.*, 1981; Wiseman *et al.*, 1985]. Epoxidation of the allyl side chain yields a 2,3-epoxide that is detoxicated to the corresponding diol by epoxide hydrolase (EH) or to the corresponding mercapturic acid derivative by glutathione transferase (GST). Intoxication *via* the epoxidation of the allyl side chain is not as significant as activation *via* the 1'-hydroxylation pathway [Luo and Guenther, 1995, 1996].

Intoxication *via* the 1'-hydroxylation pathway relies on formation of the labile sulfate conjugate (See Figure 1). The unstable sulfate ester hydrolyzes to form a reactive electrophilic intermediate (carbonium ion or quinonium cation) that binds hepatic proteins and hepatic DNA. The formation of protein and DNA adducts is dose-dependent [Drinkwater *et al.*, 1976; Miller *et al.*, 1982, 1983; Swanson *et al.*, 1981; Boberg *et al.*, 1983; Gardner *et al.*, 1995, 1996]. Studies on the inhibition of the sulfate conjugation pathway [Boberg *et al.*, 1983] and *in vivo-in vitro* unscheduled DNA synthesis (UDS) assays of estragole or methyl eugenol and their 1'-hydroxy metabolites [Chan and Caldwell, 1992; Caldwell *et al.*, 1992] provide substantial evidence that the sulfate ester of the 1'-hydroxy metabolite is the principal hepatotoxic metabolite in animals.

Figure 1
Metabolism of Estragole in Animals



*Excerpted from Smith *et al.*, 2002

Pharmacokinetic Data

Approximately 70% of a 100 microgram dose of ¹⁴C-methoxy-labeled estragole given by gelatin capsule to humans (2) was recovered within 48 hours, the majority of which was recovered in the urine (35% eliminated after 8 hours, 49.4% after 24 hours, and 61.2% after 48 hours), and remainder recovered in expired air (greater than 11% eliminated after 8 hours) [Sangster *et al.*, 1987].

In nine fasted human volunteers, ingestion of ginger snap cookies containing approximately 216 micrograms methyl eugenol (3.7 micrograms/kg bw) resulted in peak serum concentrations of 25-100 pg/g (approximately 0.000025-0.00010 micrograms/ml) with a mean of 16 pg/g [Masten, 2000]. A similar mean serum level (24 pg/g) was measured in 209 adults reported in an NHANES III U.S. survey. Over 98% of those surveyed containing detectable levels of methyl eugenol [Barr *et al.*, 2000].

Serum levels in humans are 10,000 times less than those measured in mice and rats exposed to intoxicating levels of methyl eugenol [NTP, 2000; Graves and Runyon, 1995].

Greater than 95% of a single dose of 200 mg/kg bw of methyl eugenol or 56-66% of a dose of 100 mg/kg bw of estragole administered to male rats *via* gavage was excreted in the urine within 24 hours [Solheim and Scheline, 1973]. When the same dose levels were administered by intraperitoneal injection, greater than 85% of the methyl eugenol dose and 77-87% of the estragole dose were excreted in the urine after 24 hours [Solheim and Scheline, 1973].

In female rats, greater than 71% of a 50 mg/kg bw oral dose of ¹⁴C-methoxy labeled estragole was eliminated in the first 24 hours with an additional 3.5% eliminated in the next 24 hours. Approximately 1% remained in the carcass at 48 hours. Approximately 38% was eliminated in the urine, 31% in expired air, and 1.3% in the feces [Zangouras, 1982]. In a dose-dependent toxicokinetic study, female Wistar rats were given dose levels

of 0.05 to 1000 mg/kg bw of ¹⁴C-estragole. At the low doses (0.05 to 50 mg/kg bw), the majority (55% on day 1 and 2.7% on day 2) of the dose was eliminated as ¹⁴C-labeled CO₂ in expired air. Urinary elimination accounted for a total 32.5% of the total radioactivity after 2 days. At higher dose levels (500 and 1000 mg/kg bw), elimination of radioactivity *via* expired air was less (29% on day 1 and 17% on day 2) and urinary elimination was greater (30% on day 1 and 29% on day 2) indicating a changeover in metabolism and elimination [Anthony *et al.*, 1987].

Rats administered either 37 or 150 mg/kg bw oral dose of methyl eugenol achieved peak plasma levels of 1.5 and 4 micrograms/ml. Plasma half-lives for methyl eugenol were 30 to 60 minutes and the area under the curve (AUC) was 97 and 225 micrograms/ml/minute at 37 and 150 mg/kg bw, respectively [Graves and Runyon, 1995].

F344/N rats (12/sex/group) were given 37 mg/kg bw by intravenous injection or 37, 75, or 150 mg/kg bw of methyl eugenol by oral intubation and blood was collected at time points up to 360 minutes [NTP, 2000]. Maximum plasma concentrations (C_{MAX}) of 0.656 to 3.84 micrograms/ml for males and 1.14 to 8.25 micrograms/ml for females were proportional to oral dose levels. Time to maximum plasma levels (T_{MAX}) was rapid (5 minutes) and independent of dose. The AUC increased linearly with dose for both males and females. The AUCs were in the range of 33.5 to 459 micrograms/ml/minute for males and 27 to 307 micrograms/ml/minute for females. Percent bioavailability also increased with dose. Bioavailability of methyl eugenol after a single oral dose was low (6% at 37 mg/kg bw and 19% at 150 and 300 mg/kg bw). Disappearance half-lives were in the range from 60-115 minutes for both sexes. Seventy-two (72) hours after oral or intravenous administration of [¹⁴C]-methyl eugenol to male rats, radioactivity was concentrated mainly in the liver (liver/blood ratio, 2-3) [NTP, 2000]. In mice given 25, 50, or 75 mg/kg bw, peak plasma levels were similar to those for rats (0.38 - 3.10 micrograms/ml for males and 0.12 - 4.4 micrograms/ml for females) and were reached in 5 minutes (T_{MAX}) in all groups except females in the 25 mg/kg bw groups which showed T_{MAX} of 15 minutes. Plasma half-lives were shorter (30 minutes) and the AUCs were

significantly lower than those recorded for rats (4.91-48.4 micrograms/ml/minute for males and 3.27-60.5 micrograms/ml/minute for females) indicating that methyl eugenol was eliminated more rapidly from the mouse.

In a second toxicokinetic study of longer duration [NTP, 2000], the pharmacokinetic profile was followed during repeated oral administration to rats and mice. Blood was taken from F344/N rats that had been treated with 37, 75, 150, or 300 mg/kg bw of methyl eugenol by gavage daily, 5 days per week for 6, 12, or 18 months. B6C3F1 mice treated at the same dose levels were monitored at 12 and 18 months. Absorption was extremely rapid in all dosed groups. Time to C_{MAX} was less than 5 minutes. Elimination from the blood was also rapid with elimination half-lives of 1-2 hours in both sexes. At 6 months, peak plasma levels (C_{MAX}) increased with increasing dose for most groups. Female concentrations (1.4-2.4 micrograms/ml) were higher than males (0.5-0.4 micrograms/ml) at the two lowest doses, but male concentrations (1.3-4.0 micrograms/ml) were higher than those (0.8-3.1 micrograms/ml) of females at the two highest doses. Generally, at the same dose levels, C_{MAX} was lower after 6 months of daily exposure than after single dose administration suggesting increased ability to metabolize methyl eugenol. Significant increases in both C_{MAX} and AUC between 6 and 12 months in the 150 and 300 mg/kg bw groups is evidence that metabolic saturation is achieved after prolonged exposure at higher dose levels. At all dose levels, females showed the AUC similar to naïve animals while males at 37, 75 and 150 mg/kg bw exhibited increased AUC suggesting enzymatic induction plays a more important role in males. An increase in the AUC with time suggests a decrease in the capacity to metabolize methyl eugenol with age [NTP, 2000].

For mice given 35, 75, or 150 mg/kg bw per day for 2 years, absorption was also rapid. C_{MAX} was reached after 5 minutes and increased with increasing dose for both male and females. Elimination half-lives increased with dose suggesting that the elimination was saturated for both sexes [NTP, 2000].

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

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

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

3.5.1 Metabolism

Approximately 39% and 46% of a 100 mg/kg bw dose of estragole given to rats by the oral or intraperitoneal route, respectively, is present in the 48-hour pooled urine as the *O*-demethylation metabolite 4-allylphenol (See Figure 1). Other metabolites accounting for 17% of the oral dose or 31% of the intraperitoneal dose include the product of epoxidation, hydration and subsequent oxidation of the terminal alcohol (3-hydroxy-3-(4-methoxyphenyl)propionic acid) of the allyl side-chain and the products of alkene isomerization, oxidation of the resulting C₃ position, and *beta*-oxidation yielding 4-methoxybenzoic acid and 4-methoxyhippuric acid. Approximately 5-10% of the dose was

excreted as the 1'-hydroxylation metabolite, 1'-hydroxyestragole [Solheim and Scheline, 1973].

A single intraperitoneal injection of 200 mg/kg bw of estragole, methyl eugenol, or safrole was given to male Wistar rats and urine was collected every 2 hours for 24 hours. Twenty-four (24) hours after treatment animals were terminated and the livers were removed. Urinary metabolites included the epoxide of the parent substance and the epoxide of the O-dealkylated metabolite (*i.e.*, *p*-allylcatechol epoxide from methyl eugenol and safrole and *p*-allylphenol epoxide from estragole). Liver homogenates showed the presence of safrole epoxide metabolites but not those of methyl eugenol or estragole. Liver microsomal preparations show the presence of the epoxide metabolite identified in the urine for all three substances [Delaforge *et al.*, 1980].

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

Formation of the 1'-hydroxy metabolite has been shown to be dose-dependent in both mice and rats [Zangouras *et al.*, 1981]. A dose-dependent increase in the urinary excretion of the glucuronic acid conjugate of 1'-hydroxyestragole occurs when dose levels of 0.05, 5, 500, 1,000 mg/kg bw of [¹⁴C-methoxy]-estragole is administered orally to rats or by intraperitoneal injection to mice. Only 0.9% of the dose is excreted in the urine of rats given 0.05 mg/kg bw while 8.0% is found at 1,000 mg/kg bw. The total production and exposure to the 1'-hydroxy metabolite increased significantly (1,224 to 255,000 nmoles/kg per day) as the dose was increased from 5 to 500 mg/kg. Conversely, the same increase in dose resulted in a decrease in *O*-demethylation from approximately

40% to 20% in both mice and rats. Thus, an increase in dose and a shift in metabolic pathways produce a marked increase in exposure to the 1'-hydroxy metabolite.

At low dose in humans, the 1'-hydroxylation pathway is of minor importance. Two male volunteers fed a gelatin capsule containing 100 micrograms [methoxy-¹⁴C]-estragole (1.5 micrograms/kg bw) excrete the bulk (72% and 67%) of the radioactivity in the urine and as exhaled CO₂ within 48 hours. Principal metabolites included those derived from *O*-demethylation and oxidative degradation of the allyl side chain (i.e., 4-methoxyhippuric acid, the glycine conjugate of 4-methoxycinnamic acid, and 4-methoxyphenyllactic acid). Urinary 1'-hydroxyestragole accounted for approximately 0.3% of the total dose [Sangster *et al.*, 1987]. The importance of *O*-demethylation pathway at low dose levels in human has also been observed for the double bond isomer, 4-propenylmethoxybenzene (anethole) [Sangster *et al.*, 1987; Caldwell and Sutton, 1988; Newberne *et al.*, 1999].

The 1'-hydroxylation pathway in rat and human liver microsomes indicate that the reaction is catalyzed predominantly by CYP2E1 and probably CYP2C6. The rate of 1'-hydroxylation of methyl eugenol varied widely in 13 human liver microsome samples (37 fold), but the highest activities in humans were similar to the activities in control rat liver microsomes [Gardner *et al.*, 1997]. Inducers of CYP-450 increased the number of methyl-eugenol-protein adducts. Auto-induction of the 1'-hydroxylation pathway was reported in hepatic microsomes of rats given 30-300 mg/kg bw per day oral doses of methyl eugenol for 5 days but not in rats given 10 mg/kg bw per day for 5 days [Gardner *et al.*, 1997].

In summary, *O*-demethylation is the principal detoxication pathway at low dose. At low dose levels, humans, mice, and rats show a similar tendency to metabolize alkoxyallylbenzene derivatives (*e.g.* estragole) by *O*-demethylation. At low dose significant amounts of estragole or methyl eugenol are *O*-demethylated, but as dose levels increase 1'-hydroxylation and epoxidation of alkoxyallylbenzene derivatives (*e.g.*

estragole) increase. Human production of 1'-hydroxy metabolite is expected to be very low levels of exposure (100 micrograms or 1.5 micrograms/kg bw) given that urinary excretion of the 1'-hydroxy metabolite accounts for less than 0.5% of urinary metabolites [Zangouras *et al.* 1981; Anthony *et al.*, 1987].

3.6 SUMMARY FOR CATEGORY ANALYSIS

At low levels of exposure, estragole undergoes metabolic detoxication primarily *via* *O*-demethylation to yield the corresponding phenol derivative that is readily excreted as the glucuronic acid or sulfate conjugate in the urine. As dose levels increase, a switch in metabolism occurs in which an intoxication 1'-hydroxylation pathway competes favorably with the detoxication *O*-demethylation pathway. Under these high-dose conditions liver toxicity is normally observed in animal studies.

4 TEST PLAN

4.1 CHEMICAL AND PHYSICAL PROPERTIES

4.1.1 Melting Point

The calculated melting point for estragole has been reported to be $-1.19\text{ }^{\circ}\text{C}$ (adapted Stein and Brown method) [MPBPVP EPI Suite, 2000]. Using a semi-quantitative method, the incremental change in experimental melting point between 4-methoxy- (21 $^{\circ}\text{C}$) and 3,4-dimethoxy-1-propenylbenzene (16-17 $^{\circ}\text{C}$) is 4-5 $^{\circ}\text{C}$. Based on these data and the fact that 3,4-dimethoxy-2-propenylbenzene exhibits a melting point of -4°C (Chemical Rubber Handbook), the melting point of 4-methoxy-2-propenylbenzene (estragole) is estimated to be approximately 1 $^{\circ}\text{C}$.

4.1.2 Boiling Point

The measured boiling point of estragole has been reported to be 216°C at 764 mm Hg [Merck Index, 1998] and 216°C at 760 mm Hg [Fragrance Materials Association]. The calculated boiling point according to the MPBPWIN program was 209.93°C at 760 mm Hg [MPBPVP EPI Suite, 2000]. Based on the consistency of these values, the boiling point of estragole is 216°C .

4.1.3 Vapor Pressure

Experimental value for vapor pressure was reported to be 1 mm Hg at 52.6°C [Stull, 1947]. The calculated vapor pressure of estragole has been reported to be 0.09 mm Hg (12 Pa) at 20°C [Fragrance Materials Association]. The vapor pressure of the isomer *trans*-anethole has been reported to be 0.05 mm Hg (6.67 Pa) at 20°C [FMA] and 0.041 (5.45 Pa) at 21°C for anethole, isomer unspecified [Daubert and Danner, 1989]. Given that the structure of estragole and anethole differ only in the position of a side-chain

double bond, similar vapor pressures are expected at 20°C. Therefore, the vapor pressure of estragole is approximately 0.09 mm Hg (12 Pa) at 20°C.

4.1.4 n-Octanol/Water Partition Coefficients

The Log KOW was calculated resulting in a value of 3.47 [KOWWIN EPI Suite, 2000] for estragole, in good agreement with the log KOW of 3.39 [KOWWIN EPI Suite, 2000] and 3.11 [Interactive Analysis LogP and LogW Predictor] reported for the isomer anethole.

4.1.5 Water Solubility

The solubility of estragole in an experimental study was reported to be 178 mg/L at 25°C [WSKOWIN EPI Suite, 2000a (Yalkowski, S.H. and Dannenfelser, R.M., 1992)]. The calculated value based on the log KOW of 3.47 was reported to be 84.55 mg/L at 25°C [WSKOWIN EPI Suite, 2000b]. The water solubility of the double bond isomer anethole was reported to be 111 mg/L at 25°C that is in good agreement with the measured value for estragole [WSKOWIN EPI Suite, 2000a (Yalkowski and Dannenfelser, 1992)].

4.1.6 New Testing Required

None.

4.2 ENVIRONMENTAL FATE AND PATHWAYS

4.2.1 Photodegradation

The calculated half-life value for estragole has been reported to be 2.36 hours [AOPWIN EPI Suite, 2000]. The short half-life of estragole is expected based on the fact that the 1'-position of the side chain is both a benzylic and an allylic position. This position is a site for rapid hydrogen abstraction by hydroxy radicals, peroxide radicals, and nitrogen dioxide radicals. Of more than 50 volatile organic compounds emitted by vegetation into the atmosphere, estragole was classified as exhibiting a relatively high rate of reactivity with hydroxyl radicals (no robust summary prepared) [Atkinson, 1990].

4.2.2 Stability In Water

No hydrolysis is possible for estragole. Estragole is expected to be stable in aqueous solution.

4.2.3 Biodegradation

The isomer of estragole, anethole, exhibited ready and ultimate biodegradability as measured by carbon dioxide production in an OECD 301B Guideline study. Anethole (mixed isomers) was 91% degraded within 28 days [Quest International Inc., 1994]. In an OECD 301B Guideline study, 3-methoxy-4-hydroxyallylbenzene is completely metabolized within 28 days (Quest, 1994a). Based on model predictions [BIOWIN EPI Suite, 2000] estragole is anticipated to be ultimately biodegradable. Although model predictions and data available for the isomer, anethole, predict that estragole should be readily biodegradable, it is recommended that estragole be subjected to a biodegradability study according to a standard OECD Guideline protocol.

4.2.4 Fugacity

Transport and distribution in the environment were modeled using Level III Fugacity-based Environmental Equilibrium Partitioning Model through the EPA EPI Suite 2000 program. The principal input parameters into the model are molecular weight (148.20), melting point (1.0 °C), vapor pressure (0.041 mm Hg), water solubility (111 mg/L at 20 °C), and log Kow (3.47). The model predicts that estragole is distributed mainly to the soil (73.4%) and water (25.1%) with less than 1% passing into the atmosphere [Mackay, 1996a, 1996b].

The significance of these calculations must be evaluated in light of the fact that estragole is a product of plant biosynthesis. Therefore, the environment produces estragole. The model does not account for the influence of biogenic production on partitioning in the environment nor does it take into account any biodegradation of background levels of estragole in the environment.

4.2.5 New Testing Required

None.

4.3 ECOTOXICITY

4.3.1 Acute Toxicity to Fish

A measured LC50 is available for the *p*-alkoxyallyl derivative, methyl eugenol. In rainbow trout and bluegill sunfish, the 96-hour LC50 for methyl eugenol was determined to be 6 mg/L (95% C.I. 4.9-7.2 mg/L) and 8.1 mg/L (95% C.I. 7.4-9.0 mg/L), respectively [Beroza *et al.*, 1975]. The acute 96-hour LC50 of anethole in fathead minnows using a continuous flow method was reported to be 7.69 mg/L [Broderius *et al.*, 1990]. Additionally, a calculated LC50 is available for estragole. The calculated 96-hour LC50 is 4.561 mg/L [ECOSAR EPI Suite, 2000].

Although the data for methyl eugenol, anethole and estragole consistently show an LC50 value of 5-10 mg/L, given the animal toxicity of estragole at high dose level, it is suggested that an LC50 be performed for estragole using a standard OECD Guideline 203 protocol.

4.3.2 Acute Toxicity to Aquatic Invertebrates

In a study following an OECD 201 protocol (Ward, 2003), estragole was reported to show an acute 48-hour EC50 and LC50 of 8.87 and 10.5 mg/L, respectively, in *Daphnia magna* under semi-static conditions. The NOEC for estragole in *Daphnia magna* is 4.73 mg/L. This value is on the same order of magnitude as that for tarragon oil containing >75% estragole. An OECD Guideline 202-I study is available for estragon oil (tarragon oil), the principal component of which is estragole (70-88%) (no robust summary for Lawrence, 1994). The 48-hour EC50 was 30.5 mg/L in *Daphnia magna* [Barth and Winkler, 2001]. The calculated 48-hour LC50 for estragole in *Daphnia magna* was reported to be 5.410 mg/L [ECOSAR EPI Suite, 2000]. This is in good agreement with the two experimental values for estragole and the 48-hour LC50 of 6.80 mg/L determined for *Daphnia magna* exposed to the 1-propenyl isomer, anethole [Broderius *et al.*, 1990].

4.3.3 Acute Toxicity to Aquatic Plants

The 72-hour acute toxicity of estragole in *Selenastrum capricornutum* was reported for a study using an OECD 201 protocol. The 72 hr EC50=2.81 mg/L based on average specific growth rate; 72-hr EC50=1.35 mg/L calculated using the number of cells/mL; 72-hr EC50= 1.01 mg/L using the area under the growth curve. The 72-hr NOEC is reported to be 0.118 mg/L based on number of cells/mL (Boeri, 2003). The 96-hour IC50 experimental value for green algae for the structurally related substance *trans*-anethole was reported to be 9.571 mg/L [Broderius *et al.*, 1990]. The calculated 96-hour EC50 for estragole in green algae was reported to be 3.681 mg/L [ECOSAR, EPI Suite, 2000]. Although the experimental IC50 value for the isomer is in good agreement with the calculated EC50 value for estragole, the calculated data should be further validated by comparison to an experimental EC50 determined for estragole. Therefore, an acute toxicity study is recommended using an OECD Guideline 202 protocol.

4.3.4 New Testing Required

None.

4.4 HUMAN HEALTH TOXICITY

4.4.1 Acute Toxicity

In rats and mice, estragole showed low oral acute toxicity with oral LD50s of 1,230-1,820 mg/kg bw for rats and 1,250 mg/kg bw for mice. Low acute dermal toxicity is reflected in an LD50 value of greater than 5,000 mg/kg bw for rabbits [Moreno, 1972a, 1972b; Jenner *et al.*, 1964].

Given the numerous studies available, additional acute toxicity tests in mammals are not recommended.

4.4.2 *In vitro* and *In vivo* Genotoxicity

4.4.2.1 *In vitro*

Extensive *in vitro* assays have been conducted on estragole and its metabolites. Estragole was negative in common strains of *Salmonella typhimurium* with and without metabolic activation [Zani *et al.*, 1991; Zeiger *et al.*, 1987; Sekizawa and Shibamoto, 1982; To *et al.*, 1982; Dorange *et al.*, 1977]. In one study [To *et al.*, 1982], a significant increase in the revertants per plate was reported for strain TA1538 in the presence of S-9 and 3'-phosphoadenosine 5'-phosphosulfate (PAPS) cofactor. The authors proposed that mutagenic response was related to the formation of the sulfate ester of an active metabolite. All other strains of *Salmonella typhimurium* were not mutagenic in assays using PAPS.

Other *in vitro* Ames assays with estragole and metabolites of estragole have produced equivocal results. Estragole was very weakly positive without metabolic activation in TA100 and positive in TA100 with activation. No effect was seen in TA98. The 2,3-epoxide of estragole and 1'-hydroxyestragole were positive in strains TA100 and TA1535, but negative in TA98 with or without S-13 metabolic activation [Swanson *et*

al., 1979]. But in a different study no evidence of mutagenicity was reported when 1'-hydroxyestragole was incubated with strains TA98 and TA100 of *Salmonella typhimurium* with and without S-13 metabolic activation. Addition of PAPS as a cofactor did not induce an increase in revertants. 1'-Acetoxyestragole was mutagenic in strains TA98 and TA100 but not in a dose-dependent manner [Drinkwater *et al.*, 1976]. Overall, estragole and its 1'-hydroxymetabolite do not appear to be mutagenic in *Salmonella typhimurium*.

Estragole concentrations of 0.001 to 0.00001 M did not induce the formation of chromosomal aberrations in V79 cells with and without metabolic activation or in primary rat hepatocytes [Muller *et al.*, 1994].

In an unscheduled DNA synthesis (UDS) study, a marked increase in UDS was reported when primary rat hepatocytes were incubated with estragole concentrations of 0.001 to 0.00001 M [Muller *et al.*, 1994]. When freshly prepared hepatocytes from Fisher F344 male rats were incubated with concentrations of estragole in the range from 0.000001 to 0.01 M, a significant increase in UDS, as much as 2.7 times control values, occurred at concentrations in the range from 0.0001 to 0.01 M [Chan and Caldwell, 1992]. Cytotoxicity was observed at concentrations in the range from 0.0001 to 0.01 M. Incubation of the 1'-hydroxyestragole showed increased UDS at concentrations greater than 0.00001 to 0.000001 M. Lactate dehydrogenase (LDH) leakage occurred at greater than 0.0001 to 0.00001 M for 1'-hydroxyestragole. The UDS activity and cytotoxicity of estragole occurred at concentrations approximately an order of magnitude greater than those for the 1'-hydroxy metabolites. Additionally, cytotoxicity was observed at slightly higher concentrations than those needed to induce UDS, although the differences were minimal. A clear non-linear relationship and threshold were established between dose for estragole or 1'-hydroxyestragole and UDS activity. Similar results were obtained for estragole in an earlier study [Howes *et al.*, 1990].

4.4.2.2 *In vivo*

Several *in vivo* genotoxicity assays are available for estragole. In an *in vivo* UDS study, hepatocytes isolated 4 or 12 hours after rats received a 500, 1000, or 2,000 mg/kg bw dose of estragole were evaluated for unscheduled DNA synthesis. Very slight increases in net grain counts were reported at the 500 and 1,000 mg/kg bw dose, but only at 2,000 mg/kg bw dose were the net grain counts greater than 5, which was the criteria for a positive result [Muller *et al.*, 1994].

In a study designed to detect DNA adduct formation of estragole and the 1'-hydroxyestragole metabolite, adult female CD-1 mice (mean weight 35 g) were given 12 micromoles/mouse (58 mg/kg) of [2',3'-³H]-1'-hydroxyestragole by intraperitoneal injection in trioctanoin and DNA adduct formation monitored over 20 days post exposure. Similarly, 9-day old male or female B6C3F1 mice (mean weight, 6g) were given intraperitoneal injections of 0.5 micromoles (14 mg/kg) of labeled estragole and sacrificed after 23 hours. Three adducts were formed by the reaction of 1' or 3' positions (*cis* or *trans* isomers) of estragole with the exocyclic amino group (N²) of deoxyguanosine. An additional adduct was formed by the reaction of the 3' position of estragole and the (N⁶) position of deoxyadenosine. Unlike adducts of aromatic amines (*e.g.*, N-acetyl-2-aminofluorene) which persist at near maximum levels of binding for several weeks, the three adducts of estragole-deoxyribonucleoside were removed rapidly from mouse liver DNA. Timed measurement of DNA adducts indicated a biphasic loss indicated by a sharp decline in one of the two major 1'-hydroxyestragole adducts followed by relatively constant levels of liver DNA adducts from days 3 to 20, suggesting excision repair [Phillips *et al.*, 1981].

In ³²P-post-labelling experiments with adult female CD-1 mice (mean weight, 25 g) a 2 or 10 mg dose of estragole was given by intraperitoneal injection and liver DNA samples were collected 24 hours later. The dose levels in this study were equivalent to 100 or 500 mg/kg bw, respectively. Estragole show binding activities higher than allylbenzene, anethole, and other allyl substituted benzene derivatives. A rapid drop in total adduct

formation occurred within 7 days after dosing and was followed by a relatively constant level over the next 140 days, an effect also observed in the previous study. The authors noted that the significant decrease in DNA adduct levels was probably related to DNA repair processes [Randerath *et al.*, 1984].

In a related ³²P-post-labelling experiment [Phillips *et al.*, 1984], newborn male B6C3F1 mice were given 0.25, 0.5, 1.0, and 3.0 micromoles of alkoxybenzene derivatives (including estragole, methyl eugenol and safrole) by intraperitoneal injection on day 1, 8, 15, and 22, respectively, after birth. Dose levels on days 1 and 22 were estimated to be approximately 27 and 35 mg/kg bw, 1'-hydroxyestragole and 1'-hydroxysafrole, respectively. Mice were terminated on days 23, 29, and 43 and their liver DNA was isolated and analyzed. Highest DNA adduct levels were measured for methyl eugenol, estragole, and safrole compared to controls or other substances tested. A significant (p less than 0.05) amount of adduct was detected at 43 days. Based on the results of a study of carcinogenic activity of these substances in the same species and strain (see Miller *et al.*, 1983 in Repeat Dose Toxicity), the authors concluded that adduct levels of at least 15 pmoles/mg of DNA at 23 days were required for statistically significant tumor formation [Phillips *et al.*, 1984]. The authors also noted that, compared to adults, newborn mice showed greater sensitivity to alkenylbenzene carcinogenicity.

4.4.2.3 Conclusions

The genotoxicity database on estragole shows no mutagenic potential in the Ames assay. In cytogenetic assays, there is no evidence of a genotoxic potential *in vitro*. *In vitro* UDS studies showed positive responses when rat hepatocytes were incubated with estragole. In an *in vivo* study, UDS was seen at the 2,000 mg/kg bw dose and very weak responses were seen at the 500 and 1,000 mg/kg bw doses. As demonstrated by the studies on DNA adduct formation, estragole forms DNA adducts when laboratory rodents are exposed to high dose levels, so it is not surprising that both substances and their 1'-hydroxy metabolites induce unscheduled DNA synthesis. In these studies, concentrations at which UDS occurs coincide with hepatocellular cytotoxicity. Under conditions of the studies,

estragole exhibits a genotoxic potential. Based on the available data, no additional genotoxicity tests are recommended.

4.4.3 Repeat Dose Toxicity

The study was designed to investigate the systemic toxicity of the test material under dietary and gavage routes of administration. The study was performed using an OECD Guideline No. 407 “Repeated Dose 28 Day Oral Toxicity Study in Rodents”. In the dietary study, the test material was administered continuously throughout the treatment period by dietary admixture to three groups each of ten male and ten female Fischer 344 F344/NHsd strain rats, for twenty-eight consecutive days, at dose levels of 1, 5 and 50 mg/kg/day. Two control groups each of five males and five females was treated with untreated diet only or untreated diet plus microencapsulated matrix (50 mg/kg bw/day).

In the gavage study, the test material was administered by gavage to ten male and ten female Fischer 344 F344/NHsd strain rats, for twenty-eight consecutive days, at a dose level of 50 mg/kg/day. A control group of five males and five females was dosed with vehicle alone (distilled water). Clinical signs, bodyweight development, urine samples, food and water consumption were monitored during the study. Hematology, urinalysis and blood chemistry were evaluated for all animals at the end of the study. Blood samples of test and control group animals were taken once prior to the start of treatment and again on Day 28. Plasma was separated and stored at approximately -20°C prior to dispatch to Dr Paul Carmichael, Imperial Collage, London. Biochemical studies on DNA and protein adduct formation and PCNA cell proliferation studies were performed on liver and forestomach tissues.

There were no unscheduled deaths during the study. One dietary female treated with 50 mg/kg/day developed a damaged tail from Day 14 onwards. Four high dose gavage males showed fur loss from Day 4 onwards and a further two high dose gavage males showed fur loss from Day 11 onwards. High dose gavage males showed a slight reduction in bodyweight gain during Week 1 of treatment. There was no effect on bodyweight in any group of males and female by either the dietary or gavage route of

administration. There were no differences between food intake or food efficiency uptake for any of the treated groups compared to controls. Hematological examination, blood chemical determinations and urine analysis revealed a slight increase in cholesterol in gavage males at the 50 mg/kg bw/d per day dose level.

Dietary animals treated with 1, 5 or 50 mg/kg/day showed a slight reduction in liver weight relative to bodyweight and absolute weight (males only). There was no treatment-related organ weight changes detected in gavage animals treated with 50 mg/kg/day. One dietary female treated with 1 mg/kg/day showed small nodules on the median lobe attached to the diaphragm of the liver. Three high dose gavage males showed fur loss and one high dose dietary female had a damaged tail. There were no further macroscopic abnormalities detected. There were no treatment-related changes detected. P³²-postlabeling experiments indicate that at detection limits of 1/10⁹ adducts, no methyl eugenol-DNA adduct are detected at 1 mg/kg bw/day and there is equivocal evidence of adduct formation at 5 mg/kg bw/d (Jones, 2003)

In a 90-day study with estragole (NTP, 2005), groups of 10 female mice each were administered 0, 37.5, 75, 150, or 300 mg/kg bw/d estragole via gavage once per day, five days per week for 93 days. Males were administered an additional dose level of 600 mg/kg bw/d. Animals were housed five per cage and fed ad libitum. Body weights and clinical observations were made weekly and on day 1 and at termination (day 93). At termination, blood was taken for clinical chemistry and haematology determinations and body and organ weights (heart, brain, liver right kidney, right testes, lungs, and thymus) were recorded. Tissues were prepared and histopathological examination was performed on a wide variety of tissues including the oesophagus, rectum, liver, bile ducts, salivary gland, stromal gland, epididymus, testes, pancreas, haemopoietic system, olfactory epithelium, and kidney.

In male mice, survival was 100% for all dosed groups except for the 600 mg/kg bw/d level. Statistically significant decreases in body weight were recorded for the 300 and 300 mg/kg bw/d dosed groups compared to that of controls. Hematology examinations

revealed decreases in erythrocytes and increases in the number of leucocytes, lymphocytes, reticulocytes, and platelets but only at the 300 and 600 mg/kg bw/d dose levels. Organ weight changes included increased relative (to body weight) liver and decreased body weight at 300 and 600 mg/kg bw/d. Histopathological examination revealed liver alterations at 300 and 600 mg/kg bw/d including oval cell hyperplasia, hepatocyte hypertrophy, hepatocyte degeneration all of which were described as being minimal or mild in severity.

Effects in female mice were less pronounced than in males. Survival was 100% for all dosed groups. Statistically significant decreases in body weight were recorded for the 150 and 300 mg/kg bw/d dosed groups compared to that of controls. Hematology examinations revealed decreases in erythrocytes and increases in the number of platelets, leucocytes, lymphocytes, and reticulocytes 150 and 300 mg/kg bw/d. Organ weight changes increased absolute and relative (to body weight) liver weight at 300 mg/kg bw/d. Histopathological examination revealed no alterations to the liver or any other organ or tissues at levels up to and including 300 mg/kg bw/d. Based primarily on the histopathologic changes, a NOAEL of 75 mg/kg bw/d and a LOAEL of 150 mg/kg bw/d was reported for the subchronic toxicity of estragole in male and female B6C3F1 mice (NTP, 2005).

In the rat 90-day gavage study (NTP, 2005), Groups of 10 male and 10 female F344/N rats each were administered 0, 37.5, 75, 150, 300, or 600 mg/kg bw/d estragole via gavage once per day, five days per week for 93 days. Animals were housed five per cage and fed ad libitum. Body weights and clinical observations were made weekly and on day 1 and at termination (day 93). At termination, blood was taken for clinical chemistry and hematology determinations and body and organ weights (heart, brain, liver right kidney, right testes, lungs, and thymus) were recorded. Tissues were prepared and histopathological examination was performed on a wide variety of tissues including the esophagus, rectum, liver, bile ducts, salivary gland, stromal gland, epididymus, testes, pancreas, hemopoietic system, olfactory epithelium, and kidney.

In male rats, survival was 100% for all dosed groups. Statistically significant decreases in body weight were recorded for the 300 and 300 mg/kg bw/d dosed groups compared to that of controls. Clinical chemistry changes were limited mainly to the 300 and 600 mg/kg bw/d groups. At 300 and 600 mg/kg bw/d increased levels of blood urea nitrogen, total protein, alanine aminotransferase, bile acid salts, and total iron binding capacity were reported. Additionally at 600 mg/kg bw/d, increases in alkaline phosphatase, bile acids/salts, and succinate dehydrogenase were reported. Hematology examinations revealed decreases in erythrocytes, hemoglobin, hematocrit, mean cell volume and platelet count and increased in the number of leucocytes, lymphocytes, and neutrophils but only at the 300 and 600 mg/kg bw/d dose levels. Organ weight changes included increased absolute and relative (to body weight) liver and kidney weight and decreased body weight and testes weight at 300 and 600 mg/kg bw/d. Absolute and relative heart weight was also increased at 600 mg/kg bw/d. Histopathological examination revealed liver alterations at 37.5 mg/kg bw/d including bile duct hyperplasia, oval cell hyperplasia, hepatocyte hypertrophy, periportal inflammation all of which were described as being minimal effects. Similar alterations at the 75 mg/kg bw per day dose level, were also described as minimal. The severity of hepatic effects at 150 mg/kg bw/d was reported to be mild while the effects at higher dose levels increased in severity (moderate and marked). At 150 mg/kg bw per day and higher dose levels, males also showed evidence of chronic hepatic inflammation, hepatocellular necrosis, oval cell hyperplasia, and hepatic periportal fibrosis. At 600 mg/kg bw/d, cholangiofibrosis was reported in one animal.

Effects in female rats were similar to those in males, but the onset and the severity of the effects were less pronounced than in males. Survival was 100% for all dosed groups. Statistically significant decreases in body weight were recorded for the 300 and 300 mg/kg bw/d dosed groups compared to that of controls. The only consistent clinical observation occurred among high dose animals who appeared gaunt during the course of the study. Clinical chemistry changes were limited mainly to the 300 and 600 mg/kg bw/d groups. At 300 and 600 mg/kg bw/d increased levels of creatine kinase, succinate

dehydrogenase, alanine aminotransferase, and total iron binding capacity were reported. Decreased serum iron was also reported at the two highest dose levels. Additionally at 600 mg/kg bw/d, increases in creatinine, total protein, and albumin were reported. Hematology examinations revealed decreases in erythrocytes, hemoglobin, hematocrit, mean cell volume, mean cell hemoglobin, and reticulocytes. Increases in the number of platelets, leucocytes, lymphocytes, monocytes, and neutrophils were reported beginning at the 75 mg/kg bw/d dose level. These changes were more pronounced at higher dose levels. Organ weight changes included decreased body weights at 300 and 600 mg/kg bw/d, increased absolute and relative (to body weight) liver at dose levels of 37.5 mg/kg bw/d and higher, increased absolute and relative lung weight at 300 and 600 mg/kg bw/d, increased thymus weights at dose levels of 75 mg/kg bw/d and greater, increased heart and right kidney weight at 600 mg/kg bw/d.

Histopathological examination revealed liver alterations at mainly beginning at the 75 mg/kg bw per day dose levels. At 37.5 mg/kg bw/d minimal bile duct hyperplasia, oval cell hyperplasia, eosinophilic foci, and sporadic periportal inflammation was reported. At 75 mg/kg bw/d, the same alterations were observed with greater incidence and severity. Also basophilic foci were reported at this dose level. At 150 mg/kg bw/d, additional alterations included histiocytic cell infiltrate, hepatocyte hypertrophy, mixed cell foci, At 300 and 600 mg/kg bw/day the severity of the effects was greater. Cholangiofibrosis was reported in one animal at 600 mg/kg bw/d. Based primarily on the histopathologic changes, a LOAEL of 37.5 mg/kg bw/d was reported for the subchronic toxicity of estragole in male and female F344/N rats (NTP, 2005).

In the NTP study (NTP, 2000) with the structurally related substance, methyl eugenol, groups of 10 male or female rats each were administered 0, 10, 30, 100, 300, or 1000 mg/kg bw/d methyl eugenol via gavage in 0.5% methyl cellulose once per day, five days per week for 14 weeks. Animals were housed individually and fed ad libitum. Body weights and clinical observations were made weekly and on day 1 and at termination. At termination, blood was taken for clinical chemistry and hematology determinations and

body and organ weights (heart, brain, liver right kidney, right testes, lungs, and thymus) were recorded. Tissues were prepared and histopathological examination was performed on a wide variety of tissues including the esophagus, rectum, liver, bile ducts, salivary gland, stromal gland, epididymus, testes, pancreas, hemopoietic system, olfactory epithelium, and kidney. The final mean body weight gains of male rats receiving 300 and 1,000 mg/kg bw/d and all the dosed female rats were significantly ($p=0.01$) less than those of the vehicle control. Liver weights in male rats dosed with ≥ 100 mg/kg bw per day and in female rats dosed with ≥ 300 mg/kg bw/d were significantly higher than those in control rats. Relative liver weights of male rats at 30 mg/kg bw per day were increased (14.08 g) compared to the vehicle controls (12.87 g) but not with respect to untreated controls (13.56 g). A significant increase in testis weight was observed in male rats receiving 1,000 mg/kg/d. Hematological examination revealed a decreased mean packed red cell volume in 300 mg/kg/d male rats and in male and female rats receiving 1,000 mg/kg/d. There were also increased platelet counts and increased alanine aminotransferase and sorbitol dehydrogenase activities in male and female rats receiving ≥ 100 mg/kg/d. Also, hypoproteinemia, hypoalbuminemia, and increased bile acid concentrations were evident in male and female rats receiving ≥ 300 mg/kg/d. An increase in the incidence of adrenal gland cortical hypertrophy and/or cytoplasmic alteration in the submandibular gland occurred in 100 mg/kg or greater male and female rats. The incidences of atrophy and chronic inflammation (chronic gastritis) of the glandular stomach mucosa were significantly increased in male and female rats administered 300 mg/kg or greater, and there was a hepatocellular adenoma in one male rat administered 1,000 mg/kg. There were no significant findings at 10 mg/kg bw/d. Based primarily on the liver weight changes in males, a NOAEL of 10 mg/kg bw/d and a LOAEL of 30 mg/kg bw/d was reported for the subchronic toxicity of methyl eugenol in male and female rats.

In the 14 week NTP study with mice (NTP, 2000), groups of 10 female mice each were administered 0, 10, 30, 100, 300, or 1000 mg/kg bw/d methyl eugenol via gavage in 0.5% methyl cellulose once per day, five days per week for 93 days. Animals were housed individually and fed ad libitum. Body weights and clinical observations were made

weekly and on day 1 and at termination. At termination, blood was taken for clinical chemistry and hematology determinations and body and organ weights (heart, brain, liver right kidney, right testes, lungs, and thymus) were recorded. Tissues were prepared and histopathological examination was performed on a wide variety of tissues including the esophagus, rectum, liver, bile ducts, salivary gland, stromal gland, epididymus, testes, pancreas, hemopoietic system, olfactory epithelium, and kidney. In mice, low survival rates were reported at the highest dose level of methyl eugenol in males and females. Mean body weight gains of male and female mice given 300 mg/kg were significantly less than those of the vehicle control. There was a statistical increase ($p < 0.05$) in liver weights in male mice dosed with ≥ 30 mg/kg bw/d and in female mice dosed with 300 mg/kg bw/d compared to those of the respective control groups. Increased incidences of cytologic alteration, necrosis, bile duct hyperplasia, and subacute inflammation were observed in the liver of 1,000 mg/kg male mice and 300 mg/kg and greater female mice. A significant increase in testis weight was observed in male mice receiving 100 or 300 mg/kg/d. There were no significant findings at 10 mg/kg bw/d [NTP, 2000]. Based primarily on the liver weight changes in males, a NOAEL of 10 mg/kg bw/d and a LOAEL of 30 mg/kg bw/d was reported for the subchronic toxicity of methyl eugenol in male and female mice (NTP, 2000).

Groups of CD-1 female mice (mean weight 24 g) were maintained on a diet containing 2,300 or 4,600 ppm estragole or 2,500 ppm 1'-hydroxy estragole for 10 months. The authors estimated that the dietary levels corresponded to an average daily intake of 150-300 and 300-600 mg/kg bw for animals on the 2,300 ppm and 4,600 ppm estragole diet, respectively, and 180-360 mg/kg bw for animals on the 1'-hydroxyestragole diet. To avoid intolerance the dietary concentration was reduced by 75% for the first 10 days and 50% for the next 10 days. The target diet was then maintained for 12 months. Survival at 20 months was slightly lower (68-70%) for estragole fed animals compared to control animals (78%). The average life span of mice given 1'-hydroxyestragole was 13.6 months compared to 18 months in controls. Body weights measured at 1, 4, and 8 months were markedly reduced at 4 and 8 months compared to controls. At 10 months, the incidence

of hepatomas was 58% for animals at 2,300 ppm estragole, 71% for animals at 4,600 ppm estragole and 56% for animals at 2,500 ppm of 1'-hydroxyestragole and 0 % in controls. Histopathological examinations revealed portal fibrosis, chronic inflammation and bile duct proliferation in addition to the tumors. Varied number of ceroid-laden histocytes and focal area of hyperplasia and megalocytosis were also reported. Four mice fed 4,600 ppm estragole had hepatic angiosarcomas [Miller *et al.*, 1983].

Additionally, CD-1 mice (male (55) and female (49)) were administered 370 mg/kg of estragole by gavage twice a week for ten doses beginning at 4 days of age. The mice were weaned at 35 days of age. Hepatomas were observed as early as 11 months. At 14 months, 73% of the males (3.5 hepatomas/mouse) and 24% of control males (0.6 hepatomas/mouse) exhibited hepatomas. The incidence of hepatomas in females (9%, 0.1 hepatomas/mouse) was not statistically different from control females (2%, 0.02 hepatomas/mouse) [Miller *et al.*, 1983]. In another part of the study, male (50) and female (50) CD-1 mice were administered a total dose of 9.45 micromoles/mouse of estragole or estragole epoxide or 1.87 micromoles/mouse of 1'-hydroxyestragole by intraperitoneal injection distributed in a ratio of 1:2:4:8 on days 1, 8, 15, and 22, respectively, of life. These doses correspond to 0.63, 1.26, 2.52, and 5.04 micromoles/mouse, respectively. The mice were weaned at 22 days of age. At 12 months, 65% of the mice receiving estragole exhibited hepatomas (1.7 hepatomas/mouse) versus 26% of controls (0.5 hepatomas/mouse) exhibited hepatomas. The incidence of hepatomas in mice given estragole epoxide (40%, 0.6 hepatomas/mouse) was not statistically different from control (26%, 0.5 hepatomas/mouse). For 1'-hydroxyestragole, 93% of the mice receiving the test substance (2.7 hepatomas/mouse) and 15% of control males (0.2 hepatomas/mouse) exhibited hepatomas [Miller *et al.*, 1983]

In a study using a hybrid strain of B6C3F1 mice, and the parent strain, C3H/He male and female mice and C57BL/6 male and female mice, the mice were given intraperitoneal injections of 1'-hydroxyestragole on days 1, 8, 15, and 22. Dose levels were 0.1 micromoles on day 1, 0.04 micromoles on days 8 and 15, and 0.08 micromoles on day 22

after birth. The levels are calculated to provide 11.7 on day 1, 18.8 on day 8, 9.3 on day 15 and 10.1 mg/kg bw on day 22, respectively. The experiment was terminated after 14 months. The first tumor-bearing mouse was observed at 10 months. At 12 months, 76% of the treated C3H/He male mice (3.0 hepatomas/mouse) and 26% of control mice (0.3 hepatomas/mouse) exhibited hepatomas. The incidence of hepatomas in C3H/He female mice, 6% (0.06 hepatomas/mouse), was not statistically different from those of control females. For C57BL/6 mice, the incidence of hepatomas in treated males was 14% (0.3 hepatomas/mouse) and was 5% (0.07 hepatomas/mouse) in control males. No hepatomas were observed in treated or control B57BL/6 female mice [Wiseman *et al.*, 1987].

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

In a NTP carcinogenesis bioassay, male and female F344/N rats and male and female B6C3F1 mice were administered methyl eugenol in 0.5% methylcellulose by gavage daily at dose levels of 37, 75, or 150 mg/kg bw per day, five days per week for 2 years [NTP, 2000]. Stop-exposure groups of rats received 300 mg/kg doses for 53 weeks followed by the vehicle only (0.5% methylcellulose) for the duration of the study. All rats at the highest dose level (150 mg/kg bw) and the stop-exposure dose level (300 mg/kg bw) died before the end of the study. Mean body weights of all dosed groups were less than those of the vehicle controls throughout the study. The incidences of liver non-neoplastic lesions in dosed groups of male and female rats were increased at 6 months, 12 months, and 2 years. There were statistically significant increases in oval cell hyperplasia, hepatocyte hypertrophy, and eosinophilic foci, at all dose levels in male and

female rats. At the three highest doses (75, 150, and 300 mg/kg bw per day) atypical focal bile duct hyperplasia, focal cystic degeneration, and mixed cell foci were observed, more in males than females. Many of the same non-neoplastic lesions of the liver were reported in the 300 mg/kg bw groups of male and female rats at both 6 and 12 months in the stop-exposure group. Non-neoplastic lesions of the glandular stomach included statistically significant increases in mucosal atrophy at all dose levels and neuroendocrine hyperplasia at the three highest dose levels in females and at all dose levels in males. There was a significant increase in the incidence of nephropathy in females at 300 mg/kg, and the incidence of renal tubule hyperplasia was greater in the greater than or equal to 75 mg/kg groups than in the vehicle control.

Liver neoplasms related to methyl eugenol exposure were reported in all dose groups and included hepatocellular adenomas and carcinomas, hepatocholangiomas, and In hepatocholangiocarcinomas. In all treated male and female rat groups, statistically significant increases (P equal to 0.049 in males and P equal to 0.017 in females at 37 mg/kg bw; P less than 0.001 for all other treated groups) in the incidence of hepatocellular adenomas and carcinomas were reported. Hepatocholangiomas and hepatocholangiocarcinomas were reported in the 150 mg/kg bw group of males (2/50, 4%) and females (3/49, 6%) and at higher incidence in the 300 mg/kg bw stop-exposure groups of males (13/50, 26%) and females (17/50, 34%). Both benign (3/50, 6%) and malignant (4/50, 8%) neuroendocrine cell neoplasms of the glandular stomach were reported in males at 150 mg/kg bw and in the 300 mg/kg bw stop-exposure group (2/49, 4.1% benign and 2/49, 4.1% malignant). The incidence of these neoplasms was much higher in females at dose levels of 75 mg/kg bw (13/50, 26% benign and 12/50, 24% malignant) and greater. In male rats, there were significant increases in the incidence of: malignant mesothelioma at 150 mg/kg; mammary gland fibroadenoma at 75 and 150 mg/kg; and fibroma of the subcutaneous tissue at 37 and 75 mg/kg. These neoplasms were not found in female rats at any dose level.

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

An extensive interpretation [Smith *et al.*, 2002] of the NTP study concludes that the study was compromised by a number of factors including malnutrition in both species, toxicity at all dose levels, gastric damage affecting the absorption, distribution, and metabolism of methyl eugenol, and the presence of infection in both sexes of mice. Also, the authors conclude that the study cannot be recognized as conclusive for carcinogenicity at lower, non-toxic dose levels of methyl eugenol. According to the authors (robust summary not included):

"The methyl eugenol bioassay was compromised by inappropriately high dose levels, administered by gavage, that cause significant hepatotoxicity, gastric damage, and malnutrition in both mice and rats. The presence of Helicobacter hepaticus in the livers of mice was also thought to have confounded the interpretation of the findings. Hepatic tumors occurred in severely damaged livers while the neuroendocrine tumors were likely to have resulted from endocrine

responses to chronic gastric damage. At dose levels of methyl eugenol at which hepatic tumors occurred in rats, non-neoplastic liver changes such as liver and hepatocyte enlargement, necrosis, chronic inflammation, periportal fibrosis and nodular or adenomatoid hyperplasia, were invariably present. Such recurrent liver damage, in particular chronic inflammation and hyperplasia undoubtedly altered methyl eugenol metabolism and may have strongly enhanced the likelihood of DNA damage, fixation of relevant DNA damage and progression of initiated/pre-neoplastic cells to cancer. Therefore, the hepatotoxicity induced by high dose levels of methyl eugenol most probably plays a very significant, if not an essential, role in the formation of hepatic tumors. If in humans, exposure to high levels of methyl eugenol were to be accompanied by recurrent liver tissue damage and hyperplasia, methyl eugenol might possibly induce liver cancer in humans. However, if dose levels of methyl eugenol in humans are less than those needed to induce hepatotoxicity (most probably somewhere in the range of 1 to 10 mg/kg bw/day), exposure of humans to such non-hepatotoxic levels can be assumed to be associated with a very low, probably zero, cancer risk.”

4.4.4 Reproductive Toxicity

Studies are available for a mixture of *p*-allylalkoxybenzene derivatives in three different species at multiple dose levels [Morgareidge, 1973a, 1973b, 1973c] and effects on reproductive organs have been reported in 90-day studies on estragole in mice and rats (NTP, 2005)

In an FDA sponsored study [Morgareidge, 1973a, 1973b, 1973c] that evaluated both reproductive and developmental toxicity parameters, the essential oil of nutmeg containing a mixture of *p*-allylalkoxybenzene derivatives {myristicin, safrole, elemicin, and methyl eugenol (10-20%)} and bicyclic terpene C₁₀H₁₆ hydrocarbons {*alpha*-pinene, *beta*-pinene, and sabinene (80-90%)} was given to pregnant CD-1 mice, Wistar rats, or golden hamsters.

In the mouse study, groups (20-21/group) of pregnant female CD-1 outbred mice were given 0, 6, 26, 120, or 560 mg/kg bw of the test material (FDA 71-28) by gavage in corn oil on days 6 through day 15 of gestation. A positive control group received 150 mg/kg bw per day of aspirin. Maternal body weights were recorded on days 0, 6, 11, 15, and 17

of gestation. Females were observed daily for appearance and behavior. Food consumption and body weight were monitored to eliminate any abnormalities that may be associated with anorexia in pregnant females. On day 17 all dams were subjected to Caesarian section and the number of implantation sites, resorption sites, live fetuses, dead fetuses, and body weight of live pups were recorded. Gestation index, mortality, number of implantation sites, number of corpora lutea, litter size and weights, sex and sex ratio of pups, and gross abnormalities to pups were reported. The urogenital tract of each dam was examined for anatomical abnormalities. One-third of fetuses of each litter underwent detailed visceral examination at 10x magnification. The remaining two-thirds were stained with alizarin red S dye/KOH and examined for skeletal defects.

The administration of up to and including 560 mg/kg bw per day of test article FDA 71-28 to pregnant mice on days 6 through 15 of gestation had no effects on nidation, reproduction, maternal survival or any measured fetal parameter. The number and types of abnormalities seen in tissues of the dam or pups of the test groups did not differ for the number and type occurring spontaneously in the positive or negative controls.

The rat and hamster studies use the same study protocol as that used for the mouse study. Adult female Wistar or golden hamsters were individually housed in mesh-bottom cages in a temperature- and humidity-controlled room. They were mated with untreated young adult males and observation of vaginal sperm plugs (rats) or appearance of motile sperm in vaginal smears (hamsters) was considered day 0 of gestation. Groups (22-23/dose) of pregnant Wistar rats were then given 0, 3, 2, 56, or 260 mg/kg bw of the test material (FDA 71-28) by gavage in corn oil daily on day 6 and through day 15 of gestation [Morgareidge, 1973c]. Groups (26-28/dose) of pregnant hamsters were given 0, 6, 28, 130, or 600 mg/kg bw of the test material (FDA 71-28) by gavage in corn oil daily on day 6 and through day 10 of gestation [Morgareidge, 1973b]. In the rats or hamster study, a positive control group received 250 mg/kg bw per day of aspirin.

The administration of up to and including 260 mg/kg bw per day of test article FDA 71-28 to pregnant rats on days 6 through 15 of gestation or administration of up to and including 600 mg/kg bw per day to pregnant golden hamsters on day 6 through day 10 of gestation had no effects on nidation, reproduction, maternal survival or any measured fetal parameter.

In the three-species study, no reproductive effects were observed when daily dose levels of up to 260 to 600 mg/kg bw of the essential oil predominantly composed of a combination of *p*-allylalkoxybenzene derivatives (10-20%) and bicyclic terpene hydrocarbons was administered daily to mice, rats, or hamsters during gestation. These dose levels correspond to dose levels of 26 to 120 mg/kg bw per day of *p*-allylalkoxybenzene derivatives.

In two 90-day gavage studies performed with estragole by the National Toxicology Program (NTP, 2005), there were not effects to reproductive organs of mice and rats monitored in the study. Given the lack of reproductive effects in the absence of significant toxicity to male and female rats and mice, it can be concluded that the reproductive NOAEL exceeds 75 mg/kg bw per day in mice and 37.5 mg/kg bw per day in rats.

Based on the results of reproductive toxicity on an essential oil containing a mixture of *p*-allylalkoxybenzene derivatives and the 90-day studies on estragole in mice and rats (NTP, 2005), no further testing on the possible reproductive toxicity of estragole is recommended.

4.4.5 Teratogenicity/Developmental Toxicity

In a developmental toxicity study with safrole, groups of 15-25 female Swiss mice were given oral doses of safrole by gavage daily at 0, 10, 50, 100, 150, or 200 mg/kg bw/day in olive oil for 8 days from days 6 to 14 of gestation. Males were untreated. Pregnant

females were sacrificed on day 18. Parameters monitored included survival of females, number pregnant on day 18, number of implantations, number and % reabsorbed, number of live fetuses, mean foetal weight, and number and % of malformations per dosed group. Malformations were further classified according to anomalies of the cranium, anterior and posterior phalangi, column vertebrate, and morphological irregularities and absence of sternbrae in untreated and treated groups. Fetal malformations of the palate, brain, limbs, and tail were also recorded. Maternal data indicated that survival among females was decreased in a dose dependent manner at dose levels of 100 mg/kg bw/day and above. The number pregnant was decreased at 100, 150 and 200 mg/kg bw/day but this paralleled survival rates. The number of implantations also decreased at dose levels of 100 mg/kg bw/day and above. Signs of maternal toxicity were recorded at dose levels equal to and greater than 50 mg/kg bw/day. The % resorptions increased at doses equal to and greater than 50 mg/kg bw/day. The mean fetal weight decreased at 50 mg/kg bw/day and above. There was no significant difference between fetal weight and survival between the controls and the 5 mg/kg/day dosed group. Although there was a statistically significant ($p < 0.001$) in malformations at the 50 and 150 mg/kg bw/day dosed groups there was no dose response. The authors noted that although there were significant signs of toxicity to dams and fetuses, there was no significant increase in malformations in the treated groups when compared to the control group. % malformations: control 9.2%, 5 mg/kg bw/day, 13.2%; 50 mg/kg bw/day, 19.4%; 100 mg/kg bw/day, 15.2%; 150 mg/kg bw/day, 19.4%

The only consistent anomalies observed in treated groups were a malformation to the anterior and posterior phalange, but there was no direct dose dependent change among treated groups. Based on these data, safrole did not cause any significant increase in malformations in mice fetuses at all administered dose levels. Maternal toxicity and fetal toxicity were noted at doses equal to and greater than 50 mg/kg bw/day (Moro et al., 1985). No maternal or fetal toxicity was observed at a dose level of 10 mg/kg bw per day.

In the FDA sponsored study discussed above [Morgareidge, 1973a, 1973b, 1973c], female pregnant CD-1 mice, Wistar rats, and golden hamsters were given dose levels of

up 560, 260, and 600 mg/kg bw, respectively, of an essential oil containing 10-20% *p*-allylalkoxybenzene derivatives and 80-90% bicyclic terpene hydrocarbons daily by gavage during gestation. Based on clinical observations and measurement of body weight gain, mortality, and evaluation of the urogenital tract of pregnant females there were no signs of maternal toxicity at any dose level in any of the three species. Based on measurements of fetal survival, fetal body weight, visceral examination of pups, and a complete skeletal examination of pups at all dose levels, there was no evidence of developmental toxicity at any dose level in any of the three species.

Additionally, a developmental study is available for the related substance, safrole. No teratogenic effects were reported when safrole was administered intragastrically to female Swiss mice from days 6-14 of pregnancy [Moro *et al.*, 1985].

Based on the lack of maternal and developmental toxicity in a teratology study with safrole (3,4-dimethylenedioxyallylbenzene), and a three-species study at multiple dose levels of an essential oil containing a mixture containing *p*-allylalkoxybenzene derivatives [Morgareidge, 1973a, 1973b, 1973c], it is concluded that estragole exhibits developmental toxicity at dose levels exceeding those producing maternal and developmental toxicity (>50 mg/kg bw per day).

4.4.6 New Testing Required

None.

4.5 TEST PLAN TABLE

Chemical	Physical-Chemical Properties					
	Melting Point	Boiling Point	Vapor Pressure	Partition Coefficient	Water Solubility	
Estragole CAS No. 140-67-0	Calc, A	A	A	Calc	A	
Chemical	Environmental Fate and Pathways					
	Photodegradation	Stability in Water	Biodegradation	Fugacity		
Estragole CAS No. 140-67-0	Calc	NA	R	Calc		
Chemical	Ecotoxicity					
	Acute Toxicity to Fish	Acute Toxicity to Aquatic Invertebrates	Acute Toxicity to Aquatic Plants			
Estragole CAS No. 140-67-0	R	A	A			
Chemical	Human Health Data					
	Acute Toxicity	Genetic Toxicity <i>In Vitro</i>	Genetic Toxicity <i>In Vivo</i>	Repeat Dose Toxicity	Reproductive Toxicity	Developmental Toxicity
Estragole CAS No. 140-67-0	A	A	A	A	R	R

Legend	
Symbol	Description
R	Endpoint requirement fulfilled using category approach, SAR
Test	Endpoint requirements to be fulfilled with testing
Calc	Endpoint requirement fulfilled based on calculated data
A	Endpoint requirement fulfilled with adequate existing data
NR	Not required per the OECD SIDS guidance
NA	Not applicable due to physical/chemical properties
O	Other

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