February 10, 2012

Dr. Ruth Lunn
Director, Office of the RoC, DNTP
National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle, NC 27709

Dear Dr. Lunn:

On behalf of the Flavor and Extract Manufacturers Association (FEMA), please find below additional information relevant to the nomination of alkenylbenzenes (selected dietary: estragole, myristicin, isosafrole) to the Report on Carcinogens.

Estragole was determined to be GRAS (Generally Recognized as Safe) by the FEMA Expert Panel in 1965 and is listed in the U.S. Code of Federal Regulations permitting use as a flavoring ingredient in food in the United States (21 CFR 172.515). In 2001, the FEMA Expert Panel re-evaluated the safety of methyl eugenol, a structurally related allyl alkoxybenzene, as a flavoring ingredient and concluded that:

> *...present exposure to methyl eugenol . . . resulting from consumption of food, mainly spices and added as such, does not pose a significant cancer risk. Nevertheless, further studies are needed to define both the nature and implications of the dose-response curve in rats at low levels of exposure to methyl eugenol . . .” (Smith et al., 2001).*

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) recently evaluated a group of allyl alkoxybenzenes, including methyl eugenol, present in foods and essential oils and used as flavouring agents (JECFA, 2009a). Their conclusions are summarized below:

> *The Committee concluded that the data reviewed on the six alkoxy-substituted allylbenzenes provide evidence of toxicity and carcinogenicity to rodents given high doses for several of these substances. A mechanistic understanding of these effects and their implications for human risk have yet to be fully explored and will have a significant impact on the assessment of health risks from alkoxy-substituted allylbenzenes at the*

concentrations at which they occur in food. Further research is needed to assess the potential risk to human health from low-level dietary exposure to alkoxy-substituted allylbenzenes present in foods and essential oils and used as flavouring agents.

Below we have summarized ongoing research to address the JECFA's and the FEMA Expert Panel's recommendations for further research. This research includes biochemical studies investigating the metabolic activation and detoxication of the reactive metabolites of methyl eugenol and its close structural relative, estragole, and the possible matrix effects in methyl eugenol and related alkoxy-substituted allylbenzene-containing naturals (e.g. basil oil) (see Biochemical Studies below). Research has also been undertaken to investigate the relationship of dose to the initiating effects for carcinogenesis (see Short-term Studies below). Although both of these research programs are not yet completed, the preliminary results are relevant to the Report on Carcinogens consideration of estragole, myristicin and isosafrole. Both programs are expected to be completed by the end of 2012.

Also presented is a short summary of a recent publication applying the margin of exposure (MoE) approach to methyl eugenol.

IOFI and FEMA would be pleased to continue to discuss this ongoing research program with the National Toxicology Program. Please contact us for further information and explanation as needed.

Sincerely,

Timothy B. Adams
FEMA Senior Scientist

Sean V. Taylor
FEMA Scientific Director

Introduction

The metabolic fate of a compound is determined by numerous factors including chemical structure, species, gender dose and genetic factors to name the most important. Above all, chemical structure, i.e. the presence of particular functional groups that are subject to metabolism, will largely determine the overall metabolic fate of a compound and quite minor structural variations may elicit major consequences for metabolic outcomes and events linked to this such as toxicity. In the present paper this is further evaluated in a detailed manner for a group of structurally related hydroxy- and alkoxy- substituted alkyl- and propenylbenzenes.

Hydroxy- and alkoxy- substituted allyl- and propenylbenzenes are important constituents of a variety of botanical based food items. These classes of compounds vary from one another by the presence or absence of alkylation of their *p*-hydroxyl substituents and/or the position of the double bond in the alkyl side chain. The present paper provides an overview of how these subtle structural variations dominate the metabolism of these important food-borne compounds ultimately influencing their toxicity and especially their genotoxic and carcinogenic potential. It is concluded that whereas the *p*-alkoxyallylbenzenes safrole, methyleugenol and estragole undergo metabolic activation to produce genotoxic carcinogens, replacement of their *p*-alkoxy substituents by a hydroxyl moiety and/or isomerisation of the alkyl chain double bond alters the metabolic fate of these important naturally occurring flavour molecules in favour of detoxification pathways, thereby eliminating bioactivation to a genotoxic carcinogenic metabolite.

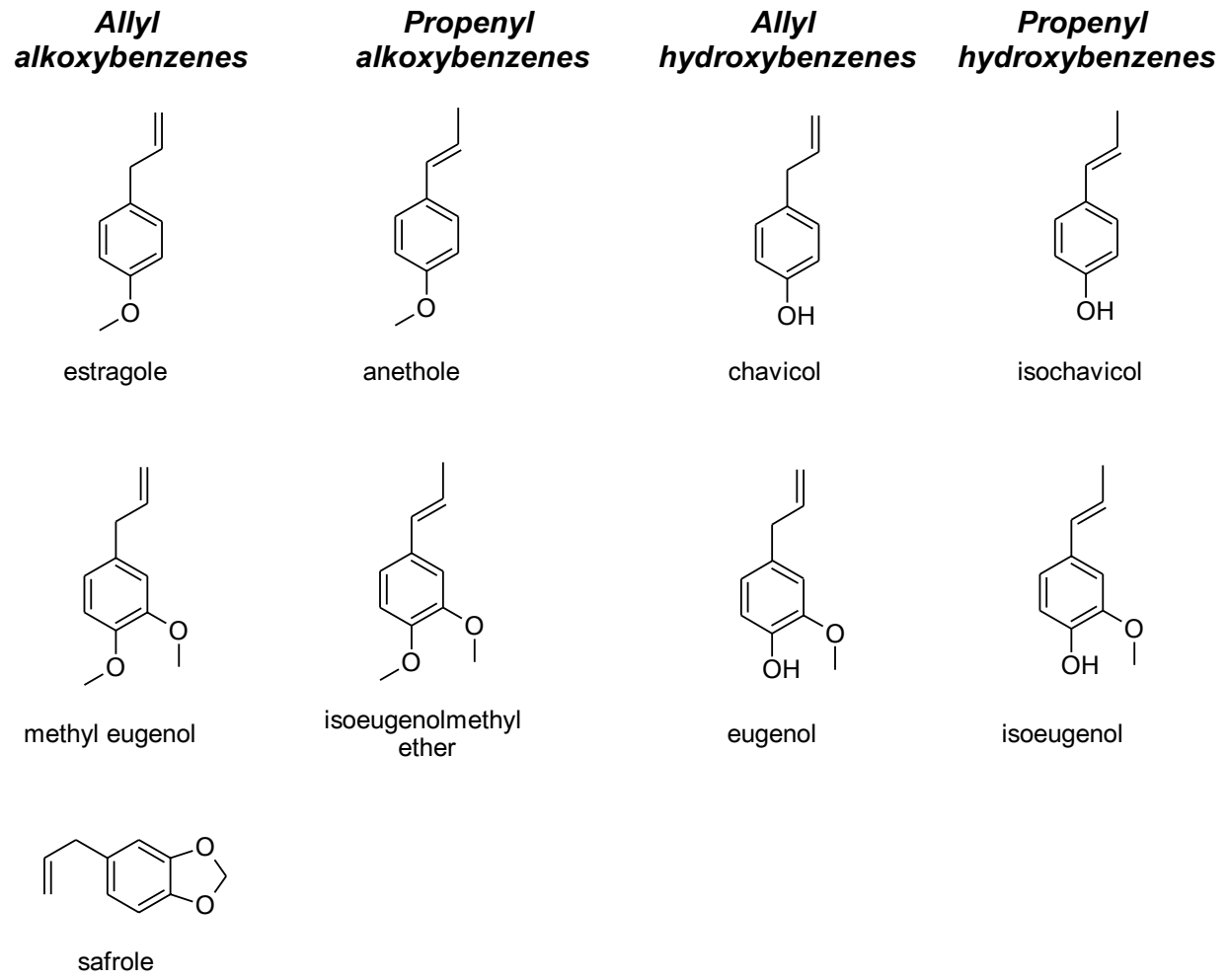
Biochemical Studies

The metabolic fate of a compound is determined by numerous factors, the most noteworthy including chemical structure, species, gender, dose and genetic factors. Above all, chemical structure, i.e. the presence of particular functional groups that are subject to metabolism, will largely determine the overall metabolic fate of a compound. Although the metabolic options for a variety of structural functional groups are well understood, nevertheless, at times, quite minor structural variations may elicit major consequences for metabolic outcomes and events linked to this such as toxicity. The capacity of minor structural changes to influence metabolic outcomes

can be illustrated by two examples. The metabolism and toxicity of the organocyanide (nitrile) function is determined by its structural attachment. When attached to an aliphatic function it is metabolised to generate hydrogen cyanide and such aliphatic nitriles that are highly toxic (Williams, 1959; Potter et al., 2001; Willhite and Smith, 1981). However, if the nitrile function is bonded directly to an aromatic ring it is metabolically stable and the organonitrile is characterised by low toxicity (Tanii and Hashimoto, 1984). Small changes in chemical structure can dramatically influence the patterns of metabolic conjugation of carboxylic acids. Furthermore, the simple carboxylic acid, benzoic acid, is metabolised in humans by conjugation with glycine to form hippuric acid (Bridges et al., 1970; Caldwell, *et al.*, 1980). However, the next homologue, phenylacetic acid, is metabolised along a quite different pathway, involving conjugation with glutamine to form phenacetylglutamine (James et al., 1972). In addition, if the phenylacetic acid acquires an alpha-methyl function (hydratropic acid) then the capacity to form amino acid conjugates is completely lost and this acid is metabolised by conjugation with glucuronic acid (Dixon et al., 1977)). In this way, quite subtile structural variations can have a major influence on metabolic and toxicity outcomes. The present paper further provides a detailed evaluation for a group of structurally related hydroxy- and alkoxy-substituted alkyl- and propenylbenzenes.

Hydroxy- and alkoxy- substituted allyl- and propenylbenzenes are important constituents of a variety of botanical-based food items. Examples of food-borne compounds belonging to these classes are the allylalkoxybenzenes estragole, methyleugenol and safrole, the propenyl alkoxybenzenes anethole (4-methoxypropenylbenzene) and isoeugenol methylether (3,4-dimethoxypropenylbenzene), the allyl hydroxybenzenes chavicol (4-hydroxyallylbenzene) and eugenol (4-hydroxy-3-methoxyallylbenzene) and the propenyl hydroxybenzenes isochavicol and isoeugenol (4-hydroxy-3-methoxypropenylbenzene). The chemical structures of these related compounds are presented in Figure 1. Table 1 presents an overview of the occurrence of these compounds in the modern food chain.

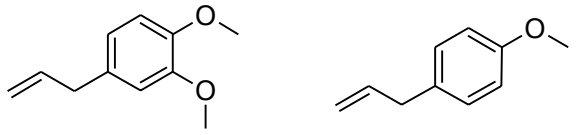
Figure 1. Chemical structures of allyl- and propenylalkoxy- and hydroxybenzenes.



The allylalkoxybenzenes safrole, methyleugenol and estragole are important constituents of herbs like nutmeg, cinnamon, anise star, tarragon, sweet basil, sweet fennel and anise vert, and are present in their essential oils as well as in food product derived from these botanicals such as pesto. These compounds have been documented to be hepatotoxic and genotoxic leading to liver tumors at high dose levels in rodent bioassays (Drinkwater *et al.,* 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987; SCF 2001a,b,c; Smith *et al.,* 2002). The related compounds eugenol, chavicol, isoeugenol, isoeugenol methyl ether and anethole, are food-borne structural analogues in which the number of alkylated hydroxyl moieties varies and/or the double bond in the alkyl side chain

is shifted. Although the principal target organ for the toxicity of all these compounds is the liver, the structural analogues do not share the genotoxicity and resulting carcinogenicity of safrole, methyleugenol and estragole. The present paper presents an overview of how these subtle structural changes impact the ultimate hazard potential of these important flavour molecules.

Recent studies evaluated the effect of dietary factors on the metabolic activation and detoxication of reactive metabolites (*i.e.,* 1'-sulfoxy metabolite) formed from methyl eugenol. *In vitro* studies have been performed to elucidate the various reactions involved in the 1'-hydroxylation, sulfation, and glucuronidation pathways of allylalkoxybenzene derivatives such as methyl eugenol. The effect of animal species and chemical structure on the involved enzyme-catalyzed reactions has been the major focus of most recent research. A very close structural relative to methyl eugenol is estragole (Figure 2).



Methyl eugenol
3,4-dimethoxyallylbenzene

Estragole
4-methoxyallylbenzene

Figure 2. The chemical structures of methyl eugenol and estragole, two alloxyallylbenzenes that are metabolized via similar mechanisms.

Estragole is a natural constituent of several herbs and spices including sweet basil. In rodent bioassays, estragole induces hepatomas, an effect ascribed to estragole bioactivation to 1′-sulfooxyestragole. *In vivo* it has been suggested that the sulfate ester of 1'-hydroxyestragole undergoes cleavage to form a reactive carbocation that, at sufficient concentration, forms protein and DNA adducts. The flavone, nevadensin (Figure 3), a basil constituent, has been identified as an inhibitor of DNA adduct formation in rat hepatocytes exposed to the proximate carcinogen 1′-hydroxyestragole (Alhusainy *et al.*, 2010). Nevadensin has been reported to be a major flavone in basil (*Ocimum basilicum*) comprising 5.6-58.4% of total flavones present (Grayer *et al.*, 1996).

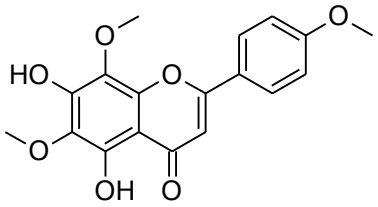


Figure 3. The chemical structure of Nevadensin (5,7-dihydroxy-6,8,4'-trimethoxyflavone).

The IC_{50} of nevadensin in primary rat hepatocytes is reported to be 0.16 ± 0.09 μM. Neither nevadensin nor 1'-hydroxyestragole demonstrated cytotoxicity as would be indicated by increased lactate dehydrogenase activity in culture supernatant. Primary rat hepatocytes exposed to 50 μM 1'-hydroxyestragole in the presence of increasing concentrations of nevadensin (0-4 μM) showed a concentration dependent decrease in DNA adduct formation (Alhusainy *et al.*, 2010). The lack of cytotoxicity in addition to the DNA adduct inhibition activity suggests that nevadensin is able to pass through the cell membrane to exert its effects. This inhibition occurs at the level of sulfotransferase (SULT)-mediated bioactivation of 1′-hydroxyestragole. The non-competitive inhibition constant (Ki) for SULT inhibition by nevadensin was 0.004 μM in male rat and human liver fractions. Concentrations of nevadensin up to 20 μM did not inhibit 1′-hydroxyestragole detoxication by glucuronidation and oxidation. This was demonstrated by no significant effect upon Km and Vmax values in the presence or absence of nevadensin in 1'-hydroxyestragole incubated with primary rat or human hepatocyte S9 fractions (Alhusainy *et al.*, 2010).

The inhibition of SULT by nevadensin was incorporated into the recently developed physiologically-based biokinetic (PBBK) rat and human models for estragole bioactivation and detoxification. The results predict that co-administration of estragole at a level inducing hepatic tumors in vivo (50 mg/kg bw) with nevadensin at a molar ratio of 0.06, representing the ratio of their occurrence in basil, results in almost 100% inhibition of the ultimate carcinogen 1′-sulfooxyestragole when assuming 100% uptake of nevadensin. Assuming 1% uptake, inhibition would still amount to more than 83%. Altogether these data point at a nevadensin-mediated inhibition of the formation of the ultimate carcinogenic metabolite of estragole, without reducing the capacity to detoxicate 1′-hydroxyestragole via glucuronidation or oxidation. These data also point at a potential reduction of the cancer risk when estragole exposure occurs within a food

matrix containing SULT inhibitors compared to exposure to pure estragole (Alhusainy *et al.*, 2010).

The carcinogenicity of allylalkoxybenzenes has been well-documented. According to NTP researchers, for example, clear evidence of hepatocellular adenomas, hepatocellular carcinomas and hepatoblastomas has been repported in B6C3F1 mice administered 37, 75, or 150 mg/kg bw /day of methyleugenol by gavage (NTP, 2000). The profile of responses was similar in rats with an increased incidence of hepatocellular adenomas and carcinomas reported in both male and female F344/N rats at dose levels (gavage) of 37, 75, 150, and 300 mg/kg bw/ day of methyl eugenol. An increased incidence of hepatocholangiomas and hepatocholangiocarcinomas were also reported at the two highest dose levels in male rats (NTP, 2000). Similar results were obtained at the NTP in a 13-week oral toxicity study, when rats and mice were dosed with 37.5, 75, 150, 300 or 600 mg/kg bw per day of estragole via corn oil gavage (NTP, 2011). The genotoxic and hepatocarcinogenic action of safrole, methyleugenol and estragole have been correlated to formation of their proximate carcinogenic 1'-hydroxy metabolites followed by sulfation to the ultimate carcinogenic 1'-sulfoxy metabolites (Borchert *et al.*, 1973; Wislocki *et al*., 1976; Wislocki *et al.*, 1977; Wiseman *et al.*, 1985; Drinkwater *et al.*, 1976; Miller *et al.*, 1976). Figures 4 and 5 provides a schematic overview of the major metabolic pathways for the allylalkoxybenzenes including this bioactivation route as well as several detoxification pathways. A major detoxification pathway of the 1'-hydroxy metabolites of the allylalkoxybenzenes in rats are conjugation with glucuronic acid catalysed by UDP-glucuronosyltransferases (UGTs), resulting in a stable metabolite which is excreted in the urine (Stillwell *et al*., 1974; Benedetti *et al*., 1974; Solheim, 1973; Anthony *et al*., 1987). In humans, however, oxidation of the 1'-hydroxy metabolite of estragole to the ketone appeared to be the major pathway for detoxification of 1'-hydroxyestragole (Punt *et al*., 2009), which appears to be a minor pathway in rats (Punt *et al*., 2008). Recent results reveal that these species-specific differences in glucuronidation and oxidation also exist for 1'-hydroxymethyleugenol and 1'-hydroxysafrole (Al-Subeihi *et al.,* submitted; Martati *et al.* submitted).

In the absence of a free phenolic function, O-dealkylation is an important detoxification pathway that competes favourably with 1'-hydroxylation of safrole, methyleugenol and estragole, especially at low dose levels in all species, particularly humans and mice (Zangouras *et al*., 1981; Sangster *et al*., 1983; Anthony *et al*., 1987). However, as dose levels increase, two

Figure 4. General routes of detoxication and intoxication for allyl alkoxybenzene flavor ingredients

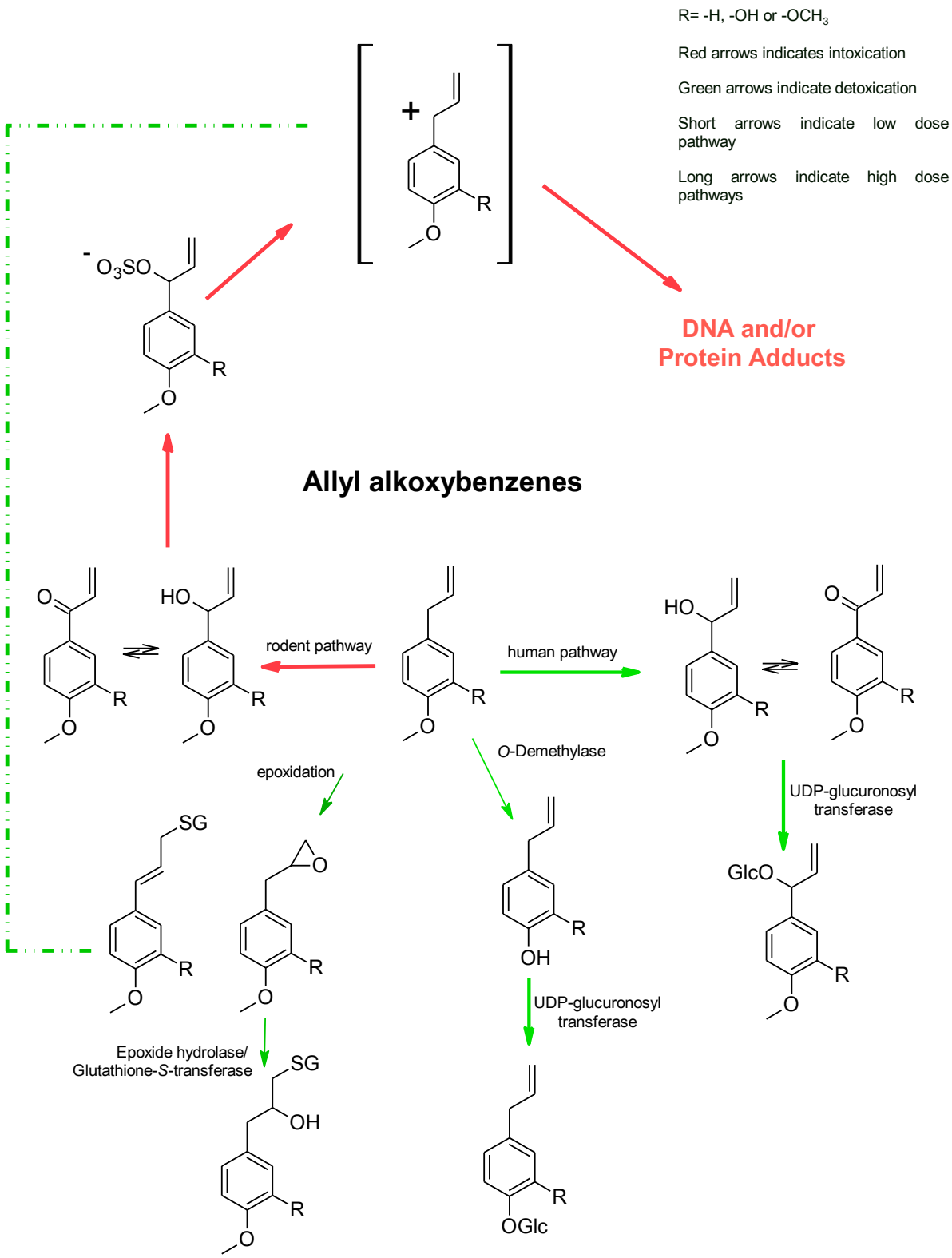
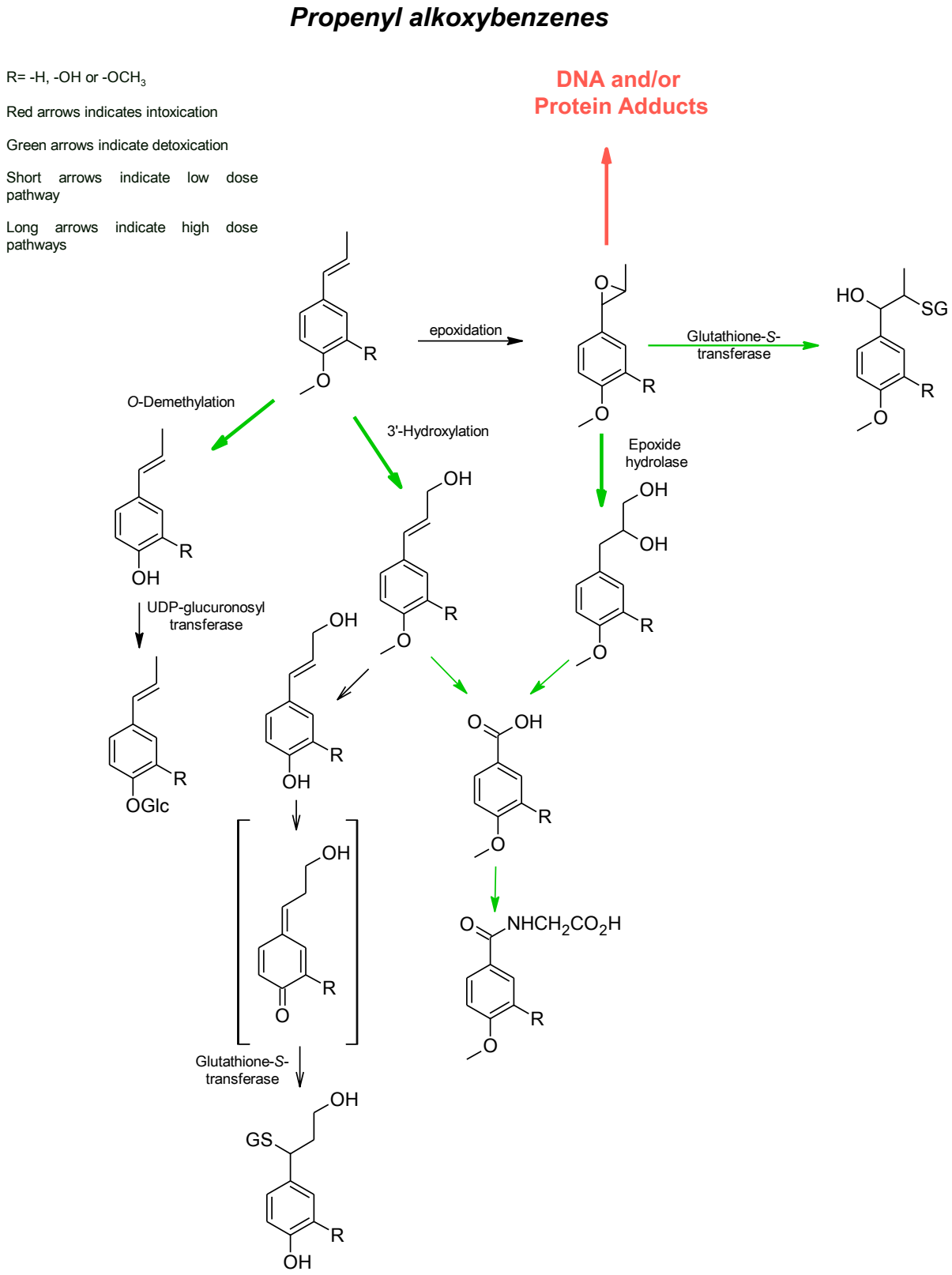
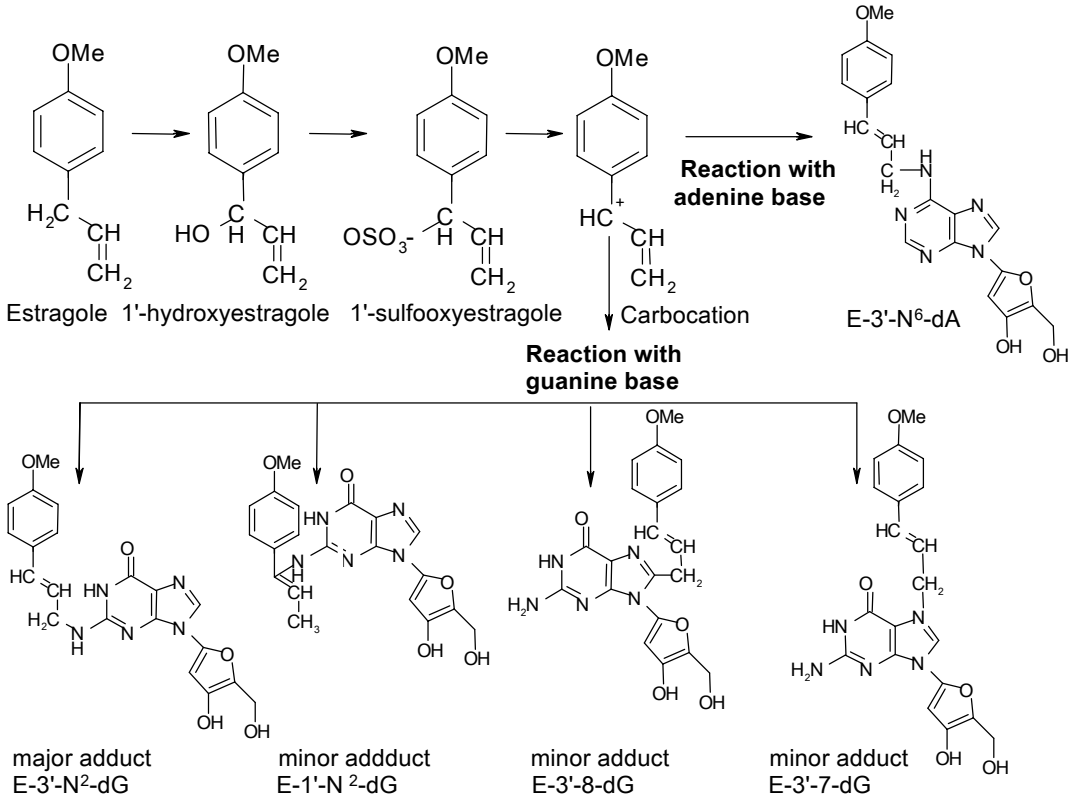


Figure 5. ***General routes of detoxication and intoxication for propenyl alkoxybenzene flavor ingredients***



bioactivation pathways compete favorably with *O*-demethylation. One pathway leads to an epoxide of the allyl double bond. This epoxide has been shown to be able to form DNA adducts *in vitro*, but these adducts are not found *in vivo* (Luo and Guenther, 1996). These results have been ascribed to the rapid detoxification of the epoxide by epoxide hydrolases and/or glutathione-S-transferases *in vivo* (Luo *et al*. 1992; Luo and Guenthner, 1995, 1996; Guenthner and Luo, 2001; Ioannides *et al*., 1981; Klungsøyr and Scheline, 1983). The other pathway, 1'-hydroxylation, discussed above, becomes a prominent pathway in rodents at dose levels >10 mg/kg bw/day (Zangouras *et al*., 1981; Anthony *et al*., 1987). As dose levels increase, saturation of the *O*-demethylation pathway and utilisation of the 1'-hydroxylation pathway provide conditions for increased formation of the downstream genotoxic metabolites that are proposed to elicit hepatocarcinogenic effects in mice and rats (Benedetti *et al*., 1977). The sulphate conjugate of the 1'-hydroxylation metabolite is currently believed to be the proximate carcinogenic metabolite for this class of compounds (Wiseman *et al*., 1987; Phillips *et al*., 1981; Phillips *et al*., 1984; Randerath *et al.*, 1984; Wiseman *et al*., 1985). The sulphate conjugate may react with DNA, RNA and proteins but can also be detoxified through conjugation with glutathione. As a result only a fraction of the 1'-sulfoxy metabolite formed is expected to result in DNA adducts. Upon reaction of 1′-sulfooxyestragole with DNA, several adducts can be formed (Figure 6). These adducts include N^2-(*trans*-isoestragole-3′-yl)-deoxyguanosine (E-3'-N^2-dG), N^2-(estragole-1′-yl)-deoxyguanosine (E-1′-N^2-dG), 7-(*trans*-isoestragole-3′-yl)-deoxyguanosine (E-3'-7-dG), 8-(*trans*-isoestragole-3′-yl)-deoxyguanosine (E-3'-8-dG), and N^6-(*trans*-isoestragole-3′-yl)-deoxyadenosine (E-3′-N^6-dA) (Phillips *et al*., 1981; Punt *et al*., 2007). The major adduct formed with the guanine base is N^2-(*trans*-isoestragole-3′-yl)-deoxyguanosine (E-3′-N^2-dG). Recently it has been reported that adducts between estragole and adenine, N^6-(trans-isoestragol-3′-yl)-deoxyadenosine (E-3′-N^6-dA) (E-3′-N^6-dA) may also be formed to a significant extent in the liver of male rats (F344) exposed to estragole at a dose level of 600 mg/kg bw for 4 weeks (Ishii *et al*., 2011). The structure of these adducts is in accordance with formation of 1'-sulfoxyestragole and the resonance stabilisation of its reactive intermediate via isomerisation of the C2'-C3-double bond to the C1'-C2' position.

Figure 6. Structures of allylalkoxybenzene purine base adducts.



Thus, the biotransformation of these substances are dose-dependent, species- and sex-specific. At low doses in rodents and humans, alkylated aromatic ring substituents are converted to phenolic derivatives that can be excreted either unchanged or as conjugates (Zangouras *et al*., 1981; Sangster *et al*., 1983, 1987; Anthony *et al*., 1987; Beyer *et al*., 2006). At similar doses, *O*-dealkylation appears to be more favoured in the mouse and human than in the rat (Zangouras *et al*., 1981; Sangster *et al*., 1983; Anthony *et al*., 1987). At higher dose levels, including those commonly used in rodent toxicity and carcinogenicity studies, biotransformation of these compounds involves greater oxidation of the allyl side chain (*i.e*., 1'-hydroxylation and alkene epoxidation) (Zangouras *et al*., 1981; Anthony *et al*., 1987). As such, when groups of rats are given increasing oral dose levels of safrole (0.9, 60 or 600 mg/kg bw), a dose-dependent change from *O*-demethylenation metabolites to allyl side chain 3'-hydroxylation (Figure 5) and/or formation of eugenol or its isomer occurs especially when the dose is raised from 60 to 600 mg/kg bw (Benedetti *et al*., 1977). In a similar study with estragole, as the dose is increased (from 0.05 to 5, 500 or 1000 mg/kg bw) in

mice and rats, the relative extent of *O*-demethylation decreases with a parallel increase in 1'-hydroxylation, especially at 50 and 1000 mg/kg bw/day (Zangouras *et al*., 1981; Anthony *et al*., 1987). The metabolic switching to a metabolic activation pathway is more significant in rats compared to mice at higher dose level (Phillips *et al.* 1981; Swanson *et al*., 1981; Miller *et al*., 1983; Wiseman *et al*., 1985). A previously developed physiologically based kinetic (PBK) model for estragole bioactivation and detoxification in male rat (Punt *et al*., 2008) corroborated that a shift from *O*-demethylation at lower doses to 1'-hydroxylation at higher doses occurs. *O*-demethylation of estragole was shown to be the major metabolic route at low doses of estragole, occurring mainly in the lung and kidney of male rat. In these organs, 4-allylphenol is formed with high affinity, reflected by a relative low Km. Due to saturation of the *O*-demethylation pathway in the lung and kidney, formation of the proximate carcinogenic metabolite 1'-hydroxyestragole, which was shown to occur mainly in the liver of male rat, becomes relatively more important at higher doses of estragole.

The effect of dose and metabolic switching on formation of the 1'-hydroxy metabolite in rats and humans provides additional insight into the carcinogenic potential of allylalkoxybenzene derivatives in both rats and humans. Rats show a clear dose-dependent increase in 1'-hydroxysafrole or its downstream metabolites in the urine. Humans show no evidence of such metabolites at low dose (1.66 mg; ~0.03 mg/kg bw) (Benedetti *et al*., 1977). At increasing doses (0.05, 5.0, 50 mg/kg bw) rats excrete 0.9%, 3.6%, and 5.5% 1'-hydroxy metabolites, respectively, whereas human volunteers given a 100 ug dose excrete only 0.3 % of the dose in the form of the 1'-hydroxy metabolite (Zangouras *et al*., 1982). Recent results revealed that this may be due to an important species difference in the way 1'-hydroxyestragole is converted in rat and human (Punt et al., 2009). In human 1'-hydroxyestragole is mainly converted to 1'-hydroxyglucuronide which can be detected in urine. In human however the major route for conversion of 1'-hydroxyestragole appeared to be oxidation to 1'-oxoestragole, indicating urinary analysis should focus on other urinary metabolites. In spite of this species difference in the pathway for detoxification of 1'-hydroxyestragole, the level of formation of 1'-sulfoxyestragole appeared to vary less than 2-fold between rat and human (Punt *et al*., 2009).

This overview of the metabolic pathways of methyleugenol and its related *p*-alkoxyalkylbenzenes reveals that bioactivation and generation of the genotoxic metabolite requires a 2,3-unsaturated allyl side chain providing an activated carbon (C_1) that is both allylic

and benzylic. The allyl side chain allows for formation of the proximate carcinogenic 1'-hydroxy metabolite and the subsequent formation of the ultimate carcinogenic 1'-sulfoxy metabolite. Also a para alkoxy substituent (OCH_3) on the phenyl ring is required to stabilize the incipient reactive intermediate that forms from the 1'-sulfoxy metabolite. The overview also suggests that once *O*-dealkylation converts an alkoxy substituent into a hydroxyl moiety, possibilities for swift conjugation and excretion may occur. The next sections evaluate to what extent modifications in the allyl side chain and/or the alkoxy subtituents in the allylalkoxybenzenes modify their metabolic fate and ultimate toxicological effects.

Modification of the allyl side chain: metabolism and toxicity of anethole and isoeugenol methyl ether

Isomerisation of the double bond in the allyl side chain of methyleugenol and estragole gives rise to isoeugenol methyl ether and anethole, respectively (Figure 1). Extensive biochemical and toxicological data (Newberne *et al*., 1999) document that the target organ for anethole toxicity is the liver and that the ultimate hepatotoxic metabolite is anethole epoxide. Anethole epoxide exhibits hepatocellular cytotoxicity at concentrations 10 times lower than those of anethole itself (Bounds and Caldwell, 1996; Caldwell, 1992; Marshall and Caldwell, 1992, 1996; Howes *et al*., 1990). Inhibitors of detoxification enzymes for anethole epoxide (i.e., epoxide hydrolase and glutathione transferase) increase the cytotoxic effects of anethole (Marshall and Caldwell, 1992). From toxicity studies, species and sexes that show the highest rate of daily production of anethole epoxide also exhibited signs of hepatotoxicity at the lowest dose levels of anethole (Newberne *et al*., 1989, 1999). Since neither anethole nor anethole epoxide are genotoxic (Nestmann *et al*., 1980; Heck *et al*., 1989; Mortelmans *et al*., 1986; Gorelick, 1995; Howes *et al*., 1990; Marshall and Caldwell, 1992, 1996; Calwell *et al*., 1992; Randerath *et al*., 1984; Phillips *et al*., 1984; Abraham *et al*., 1996; Marzin *et al*., 1979; Al-Harbie *et al*., 1995), the hepatotoxic effects and neoplastic effects observed at high dose levels in studies with laboratory animals result from a non-genotoxic mechanism in which toxicity is the result of continuous elevated hepatocellular concentrations of anethole epoxide. Quantitative data on the conversion of anethole to anethole epoxide can be related to hepatotoxic effects observed in these short-term and long-term rodent studies (Truhaut *et al*., 1989).

Figure 4 presents an overview of the metabolic pathways for anethole. Metabolic studies have shown that at low dose levels (<5 mg/kg bw/day) *trans*-anethole is rapidly absorbed and detoxified in rodents primarily by O-demethylation and in humans primarily by 3'-hydroxylation- (Bounds and Caldwell, 1992, 1996; Sangster *et al.*, 1987; Caldwell and Sutton, 1988). In humans, efficient detoxification of *trans*-anethole by 3'-hydroxylation and subsequent β-oxidation yields conjugated benzoic acid derivatives which are rapidly excreted in the urine (Sangster *et al.*, 1987; Caldwell and Sutton, 1988). At these dose levels, bioactivation and toxicity *via* epoxidation is a minor pathway and is quantitatively similar among mice, rats, and humans with the rat showing a slightly greater tendency to epoxidise compared to the mouse or human.

From this overview it follows that the isomerisation of the double bond in the allyl side chain shifts the regioselectivity of the cytochrome P450 mediated hydroxylation of the chain resulting in a 3'-hydroxy metabolite instead of a 1'-hydroxymetabolite. This not only eliminates the chances of formation of the proximate carcinogenic metabolite, but also shifts the metabolism to a detoxification pathway, because the 3'-hydroxymetabolite readily undergoes hydroxyl group oxidation followed by β -oxidation that yields conjugated benzoic acid derivatives which are rapidly excreted in the urine (Sangster *et al.*, 1987; Caldwell and Sutton, 1988).

The inclusion of an additional methoxy substituent at the *meta*-position in isoeugenol methyl ether increases metabolic options for detoxification which reduces endogenous levels of the corresponding epoxide (Solheim and Scheline, 1976). Male albino Wistar rats administered a single oral dose of 200 or 400 mg isoeugenyl methyl ether/kg bw formed greater than 77% urinary metabolites principally via 3- and 4-*O*-demethylation and 3'-hydroxylation. Metabolites resulting from the epoxidation pathway account for less than 1% of the urinary metabolites. The low production of the corresponding epoxide correlates with the lack of reported hepatotoxic effects for isoeugenyl methyl ether.

Taken together, the data suggest that at low levels of exposure, *trans*-anethole and isoeugenyl methyl ether are efficiently detoxicated in rodents and humans. Chronic exposure to high dose levels in female rats, results in the continuous daily production of hepatocellular concentrations of anethole epoxide that induce a continuum of cytotoxicity, cell proliferation, cell death, and following prolonged exposure, liver tumors. Quantitatively, daily conversion of

anethole to the epoxide in 2 year study of female rats (Truhaut *et al*., 1989) resulted in an increase in the incidence and severity of hepatotoxicity was in the range of 30 to 120 mg/kg bw per day (15% epoxide metabolites at an anethole dose of 200 mg/kg bw per day and 23% epoxide metabolites at anethole dose of 550 mg/kg bw/day) (Newberne *et al*., 1999; Bounds, 1994). At the highest dietary level of anethole (550 mg/kg bw per day) female rats exhibited both hepatic toxicity and hepatic tumors, but at dietary levels of 120 mg/kg bw per day of anethole (predicted to generate approximately 22 mg anethole epoxide/kg bw per day), there was no evidence of hepatotoxicity. In female rats showing no hepatotoxic effects, the lifetime body burden to anethole epoxide was approximately 17,000 mg/kg while in humans, lifetime body burden of anethole epoxide resulting from intake of anethole (0.002 mg/kg bw per day) is approximately 51 mg/kg. These numbers reveal a large margin between exposure levels causing hepatotoxicity in experimental animals and levels expected to occur in realistic human exposure scenarios.

Thus, comparison of the metabolic and toxicity profile of anethole and isoeugenyl methyl ether to that of the related allylalkoxybenzenes safrole, methyleugenol and estragole, reveals that the isomerisation of the double bond in the allyl side chain significantly alters the chemical characteristics and thus the metabolism favors detoxification, eliminating formation of the proximate genotoxic 1'-hydroxymetabolite and resulting in a different mode of toxic action.

Modification of the alkylation state of the hydroxyl substituents: metabolism and toxicity of chavicol and eugenol

O-Dealkylation of a methoxy substituent in methyleugenol and estragole gives rise to eugenol and chavicol, respectively (Badger *et al*., 1999; Zangouras *et al*., 1981; Figure 1). Thus, the difference in toxicity between methyleugenol and eugenol can only be associated with the presence of a free hydroxyl moiety in eugenol. The free phenolic function implies that conjugation becomes a primary mode of metabolism and excretion. Figure 2 presents the metabolic pathways for allylhydroxybenzenes, also representative for eugenol, and reveals that additionally eugenol undergoes 1) isomerization to yield isoeugenol that may then undergo 3'-hydroxylation and reduction of the double bond, 2) epoxidation of the allyl double bond to yield an epoxide that hydrolyzes to the corresponding diol which then may be oxidized to the corresponding lactic acid derivative, 3) conjugation of its quinone-methide type intermediate

with glutathione, and 4) hydroxylation at the allyl position to yield 1'-hydroxyeugenol. Since all of these metabolites possess a free phenolic OH group or other polar oxygenated functional groups, they readily conjugate with glucuronic acid or sulphate and are excreted in the urine (Fischer *et al*., 1990). In human experiments, 95% of an ingested dose of 0.6 mg or 150 mg eugenol was excreted in conjugated form in the urine within 24 hours (Sutton, 1986; Fischer *et al*., 1990). Within 24 hours, greater than 55% of a 150 mg oral dose of eugenol administered in gelatine tablets after consumption of a normal breakfast was excreted in the urine as the glucuronic acid or sulphate conjugates of eugenol. Other conjugated urinary metabolites included *cis*- and *trans*-isoeugenol (7%) formed by isomerization of the double bond, 3-(4-hydroxy-3-methoxyphenyl)propane formed by reduction of either eugenol or isoeugenol, and 3-(4-hydroxy-3-methoxyphenyl)propionic acid (4.6%) presumably formed by allylic hydroxylation of isoeugenol followed by NADH-dependent enzymatic reduction of the double bond. Conjugated metabolites formed from epoxidation of eugenol included eugenol epoxide (1.6%), the corresponding diol (3%), and 2-hydroxypropionic acid derivative, 3-(4-hydroxy-3-methoxyphenyl)propionic acid (3.3%) formed by oxidation of the diol primary alcohol. Tentatively identified metabolites included a thiophenol (11%) metabolite presumably formed by glutathione conjugation at an aromatic ring position and a trace amount (<1%) of 1'-hydroxyeugenol from by allylic hydroxylation of the benzylic position. The authors concluded that eugenol is subject to rapid first pass conjugation and rapid elimination accompanied with only a small fraction participating in isomerization, epoxide-diol, glutathione conjugation or 1'-hydroxylation pathways (Fischer *et al*., 1990).

At lower dose levels, conjugation predominates. Over 85% of the urinary radioactivity of 0.6 mg dose of [^{14}C]-eugenol given to two volunteers was excreted after 24 hours in the urine as glucuronic acid and sulfate conjugates of eugenol with the glucuronic acids predominating. Essentially all (95 and 103%) of the radioactivity was excreted in 24 hours. Minor amounts (2% each) of the corresponding diol 3-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol and alcohol 3-(4-hydroxy-3-methoxyphenyl)propane-2-ol were also detected (Sutton, 1986). Results of studies in rodents indicate that eugenol has a metabolic fate similar to that in humans. In a dose-dependent metabolism study, female Wistar rats were administered 0.5, 5, 50 or 1000 mg/kg bw of ring labelled [^{14}C]-eugenol in trioctanoin by stomach tube. At 24 hours, the urinalysis revealed glucuronic acid and sulphate conjugates of eugenol, the *O*-demethylation metabolite 3,4-

dihydroxypropylbenzene and the reduced metabolite 3-methoxy-4-hydroxypropylbenzene (Sutton *et al*., 1985; Sutton, 1986). At low doses (0.5, 5, and 50 mg/kg bw), sulphate conjugates were the principal metabolites while at the highest dose (1000 mg/kg bw) glucuronic acid conjugates predominated. Metabolites resulting from reduction or *O*-demethylation (*i.e*., 3,4-dihydroxypropylbenzene and 3-methoxy-4-hydroxypropylbenzene) were not detected at the high dose in the 24-hour urine.

As outlined above, based on experiments in humans, it can be concluded that the majority of orally administered eugenol is conjugated with glucuronic acid or sulphate and excreted in the urine. Minor metabolic pathways include oxidation to form the corresponding epoxide followed by hydrolysis to the diol and then further oxidation products; isomerization followed by allylic oxidation and then reduction; reduction of the alkene; or 1'-hydroxylation (trace). All these metabolites possess a free phenolic hydroxy moiety and are readily conjugated and excreted in the urine.

The increased possibilities for swift conjugation and excretion due to the free phenolic hydroxyl moiety can explain why eugenol is not a genotoxic carcinogen, in contrast to methyleugenol (NTP, 2000; Maronpot *et al.*, 1987). In studies with eugenol, there was no significant increase in the incidence of hepatocellular neoplasms in either male or female B6C3F$_1$ mice in 2-year bioassay study at any dose level (Maronpot *et al.*, 1987) and there was no evidence of an increased incidence of hepatocellular neoplasms in male or female F344/N rats (NTP, 1983) at dietary levels calculated to provide a daily intake of 150, 300, or 625 mg eugenol/kg bw/ day (FDA, 1993). The swift detoxification of eugenol, due to its free phenolic hydroxyl, is entirely consistent with a lack of any significant toxicologic and carcinogenic responses. The short residence time of eugenol in the body explains the lack of toxic effects observed in numerous studies on eugenol.

Modification of both the allyl side chain and the alkylation state of the hydroxyl substituents: metabolism and toxicity of isoeugenol

Combined isomerisation of the allyl side chain and replacement of the *p*-methoxy substituent by a *para*-hydroxyl moiety converts methyleugenol to isoeugenol (Figure 1). In isoeugenol the presence of a hydroxyl substituent at the *para*-position in combination with the shift in the double bond in the allyl side chain modifies the metabolic options and resulting toxicity

compared to methyleugenol. Conjugation of the free phenolic function with sulfate and glucuronic acid is the predominant detoxification pathway for isoeugenol. Greater than 85% of a 156 mg [^{14}C]-isoeugenol/kg bw administered by gavage to male Fischer-344 rats was excreted as the glucuronic acid and sulfate conjugate in the urine within 72 hours (Badger *et al.,* 1999). Likewise, 82% was excreted as the same urinary conjugates 72 hours after 15.6 mg [^{14}C]-isoeugenol/kg bw, administered by intravenous injection. Following both administration routes, approximately 10% of the administered dose was excreted in the faeces and less than 0.1% was recovered as expired CO_2 with less than 0.25% of the dose remaining in selected tissues.

Although the 2-year NTP bioassay (NTP, 2010) in mice showed some evidence of increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined) at oral gavage doses of 75, 150, or 300 mg/kg bw/ day of isoeugenol. This response was not dose-related and was only reported in male B6C3F1 mice, a sex and species with historically high levels of background hepatocellular neoplasms (Maronpot *et al.*, 1987). From a biological perspective, the increase in the tumor incidence in male B6C3F$_1$ mice reflects the impact of high-dose liver damage to an organ already prone to spontaneous development of liver neoplasms (Haseman *et al*., 1986, 1989). The lack of any hepatotoxic or hepatocarcinogenic effects in female mice or either rat sex support the conclusion that isoeugenol does not present a carcinogenic hazard to humans when consumed at regular dietary intake levels estimated to be 45 µg/day in Europe and 26 µg/day in the USA which is equivalent to 0.75 and 0.43.mg/kg bw/day in Europe and the USA respectively (EFFA, 2004; Gavin et al., 2008). And the lack of toxicity even at relatively high levels of exposure is due to conjugation, 3'-hydroxylation, and β -oxidation pathways that provide efficient routes of detoxification reducing the toxic potential as compared to that of methyleugenol.

Although the 2-year NTP bioassay (NTP, 2010) in mice, showed some evidence of increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined) at oral gavage doses of 75, 150, or 300 mg/kg bw/ day of isoeugenol, the response was not dose-related and it was reported only in male B6C3F1 mice, a sex and species with historically high levels of background hepatocellular neoplasms (Maronpot *et al.*, 1987). From a biological perspective, the increase in the incidence of tumors in male B6C3F$_1$ mice reflects the impact of high-dose liver damage to an organ already prone to spontaneous development of liver neoplasms (Haseman *et al*., 1986, 1989). The lack of any hepatotoxic or

hepatocarcinogenic effects in female mice or male or female rats support the conclusion that isoeugenol does not present a carcinogenic hazard to humans when consumed at regular dietary intake levels and the lack of toxicity even at relatively high levels of exposure is due to conjugation, 3'-hydroxylation, and β -oxidation pathways that provide efficient routes of detoxification reducing the toxic potential as compared to that of methyleugenol.

Short-term Studies

Although it has been demonstrated (JECFA, 2009b) that a linear relationship exists between DNA adduct formation and dose level of methyl eugenol, no comprehensive study of the relationship of dose to the initiating effects for carcinogenesis have been investigated. To study initiating effects of methyleugenol in rat liver, groups of male F344 rats (8/group) were administered methyl eugenol by intragastric instillation in 0.5% methylcellulose, at dosages of 0, (C, control groups 1 and 2), 62 (LD, low-dose groups 3 and 4), 125 (MD, mid-dose groups 5 and 6), or 250 (HD, high-dose groups 7 and 8) mg/kg bw/dose, 3 doses per week for 8 and 16 weeks (equivalent to dosages of 37, 75 or 150 mg/kg bw/day, 5 days per week as in the National Toxicology Program; see table 1). At 8- and 16-week intervals of initiation, 3 rats from each dose level (groups 1, 3, 5 and 7) were killed and samples of liver and blood (16 weeks only) were taken for analysis of blood liver enzymes, DNA adducts, cell proliferation (liver only) and hepatocellular altered foci (HAF, liver only) (Private Communication to IOFI, 2010).

Table 1. *Comparative cumulative doses*

14-Week study [a]		16-Week study [b]	
Individual doses (mg/kg bw/dose)	Cumulative doses (mg/kg)	Individual doses (mg/kg bw/dose)	Cumulative doses (mg/kg)
10	700	NA	NA
30	2100	62	3000
100	7000	125	6000
300	21000	250	12000
1000	70000	NA	NA

[a], NTP, 2000; Abdo *et al.*, 2001; Herbert and Abdo, 2003: The daily doses (5 doses per week) were 10, 30, 100, 300, or 1000 mg/kg bw in this 14-week study with male and female rats. This amounted to 50, 150, 500, 1500 or 5000 mg/kg bw/wk. 7000 mg/kg produced 1/10 hepatocellular adenoma in one male rat; from this study 185, 375 or 750 mg/kg bw/wk were chosen as the doses of the bioassay;

[b], present study: Based on the above study, the 3 doses were selected, i.e., 62, 125 or 250 mg/kg bw/dose, 3 doses per wk, or 185, 275 or 750 mg/kg bw/wk;

NA, not applicable.

No compound-related deaths or clinical findings occurred in the study. No changes were evident in any of the serum liver enzymes monitored at the 16-week interval.

At all 3 dose levels administered, methyl eugenol produced a reduction in body weight gain, which was statistically significant ($p=0.005$) at 8 weeks in the two highest doses. This significant reduction represented 12% and 21% of the MD and HD, respectively. The reduction in body weight gain and relative liver weight increase ($p=0.011$) is in accordance with the literature for similar cumulative doses (NTP, 2000; Abdo *et al.*, 2001; Herbert and Abdo, 2003). Moreover, the consequences of these 2 findings led to hypoproteinemia (Abdo *et al.*, 2001), which was not monitored in the present study. In addition, with protein catabolism of this magnitude, i.e., 21% decrease in body weight gain at 8 weeks, a shift in energy homeostasis is usually produced in which, after exhaustion of carbohydrates, fat cannot be utilized as an energy substrate, especially in the liver (Matthews and Battezzati, 1994; Williams and Iatropoulos, 2002). Nevertheless, by week 16, equilibrium was reestablished, as reflected by the fact that the mean body weight gains and terminal mean body weights had recovered.

At 8 weeks, in MD and HD groups there were group mean body weight gain reductions ($p=0.005$), group mean body weight reductions ($p=0.005$), and increases in group mean relative liver weight ($p=0.011$). ^{32}P-Nucleotide postlabelling (NPL) revealed 3 adducts (2 major and one minor) in the livers of all dosed groups. The mean hepatic DNA-adduct value of the MD and HD groups were significantly increased compared to control and LD groups. Proliferating cell nuclear antigen (PCNA) immunohistochemistry revealed that the mean group hepatocellular replicating fraction (RF) in all methyl eugenol dosed groups was increased ($p<0.01$) compared to controls, as was the hepatocellular hypertrophy, karyomegaly and multinucleation, Kupffer cell activation, and enlargement of the periportal cholangiole plexus. Only in the HD rats were HAF identified by placental-type (π) glutathione s-transferase (GST-P) immunohistochemistry.

At 16 weeks, there were no body weight changes, or terminal body weight changes when compared to controls, respectively. All methyl eugenol groups had dose-related increases ($p=0.05$ to $p=0.005$) in mean DNA-adduct values in both liver and WBCs compared to controls, although the WBC levels were approximately 100 times less than the liver. The mean group

hepatocellular RF in all methyl eugenol groups was significantly ($p<0.001$) increased compared to controls, as was the incidence and severity of the diffuse hepatocellular hypertrophy, karyomegaly and multinucleation, Kupffer cell activation, and enlargement of the periportal cholangiole plexus. All methyl eugenol groups had HAFs in a dose-related pattern.

Three DNA adducts, two major and one minor, were found in the liver, even in the LD group. However, not having reference standards, it was not possible to say with certainty that these DNA adducts were identical to those reported in cultured human hepatocytes (Zhou *et al.*, 2007), or F344 rat liver (Ellis, 2007). At 8 weeks, the mean liver DNA adduct values of MD and HD groups were statistically higher ($p<0.001$) compared to both groups 1 (control) and 2 (LD methyl eugenol). This is the first time that DNA adducts are reported with adult F344 rat livers. At 16 weeks, the LD and MD methyl eugenol groups were significantly elevated ($p=0.05$), with the HD group assuming a significance at $p=0.005$. In addition, the DNA adduct levels were higher. With WBC at 16 weeks, likewise all 3 methyl eugenol groups were significantly elevated compared to controls. Here the adduct levels present were about 1% of the adducts found in the liver. This may be a useful biomarker, as WBC DNA can be readily obtained and monitored in humans.

The low levels of DNA adducts in control samples probably reflects exposure of controls, which is known to occur (Samsone and Fox, 1977; Nicholls *et al.*, 2005). This could be a consequence of the relative volatility of methyl eugenol, despite the fact that the control cages had a special cover.

The mean RF values of all 3 methyl eugenol groups were significantly increased in a dose-and-time-related pattern, compared to controls. Moreover, comparing the 2 interim sacrifice mean RF values of all methyl eugenol groups, the 16-week values were lower, indicating that the hepatocytic compensatory (adaptive) response was completed and was receding. This is again similar to the BrdU-monitored hepatocellular proliferation response (Abdo *et al.*, 2001; Herbert and Abdo, 2003). The recession of proliferation suggests that with further dosing methyl eugenol would have negligible promoting activity through this mode of action.

At both sacrifice intervals, the methyl eugenol liver findings, using hematoxylin and eosin and GST-P histopathology comprised several dose-and-time-related changes. A diffuse hepatocellular hypertrophy, with sporadic karyomegaly and binucleation, with concomitant

activation of Kupffer cells with pigment was present. Also, in periportal fields, there was an enlargement of the cholangiole plexus expanding the canals of Herring, which give rise to cholangiocytes-lined cholangioles. In the PCNA stained slides the PCNA positive cholangiocytes and neighboring pluripotential oval cells were evident in a dose-related increasing pattern, while they were absent in the controls. This reflects an adaptive compensatory response to increased hepatobiliary metabolic demands resulting from methyl eugenol dosing because the rat does not have a gall bladder to gauge the intrahepatic biliary homeostasis (Williams and Iatropoulos, 2002; Greaves, 2007). Thus, the enlarged plexus is not the same as the "bile duct hyperplasia", which was described previously, at comparable cumulative doses (Abdo *et al.*, 2001; Herbert and Abdo, 2003). Bile duct hyperplasia can be viewed as a preneoplastic precursor of hepatobiliary neoplasia (e.g. furan, NTP, 1993), but this neoplasia did not occur in the 2-year methyl eugenol study (NTP, 2000).

Starting with the 8-week interval, the HD methyl eugenol rats had mixed foci of altered hepatocytes (HAF), which were GST-P positive. The incidence of GST-P positive HAF value was 0.51 ± 0.3, and the severity was mild. This incidence value was significant ($p<0.01$) compared to all other groups. At 16 weeks, the incidence and severity of HAF was increased in a dose-related manner in all methyl eugenol groups. The incidence of GST-P positive values of 1.26 ± 0.24 was significantly ($p<0.01$) increased compared to all other groups. The average diameter of each HAF lesion has increased from ~ 204 μ in the LD and MD groups, to ~ 918 μ in the HD group. This is in accordance with the previously published literature (Abdo *et al.*, 2001; Herbert and Abdo, 2003). The induction of HAF reflects development of promotable preneoplastic lesions, which we expect will be promoted with phenobarbital in the promotion phase (for 24 weeks) of this study. However, compared to an activation-dependent DNA-reactive hepatocellular carcinogen, 2-acetylaminofluorene (AAF), at both carcinogenic and noncarcinogenic doses (with PB promotion) in male F344 rats, the GST-P positive HAF values at 12 weeks in the HD group at a cumulative dose of 12000 mg/kg methyl eugenol were 15 times lower with methyl eugenol compared to AAF given at a cumulative dose of 94 mg/kg (Williams *et al.*, 1998; 2004). At this phase of the study, the initiating activity of methyl eugenol can be considered weak.

Thus, biomarker changes of initiation were produced by 8 and 16 weeks of dosing with methyl eugenol. One half of the remaining groups (3, 5, 7) of rats are currently being exposed to

500 ppm phenobarbital, in the diet, to promote hepatocellular neoplasia at 24 weeks of dosing, with the other half of the groups (6, 4, 8) being on recovery for 24 weeks.

At 8 weeks, the high dose methyl eugenol rats only had mixed foci of altered hepatocytes (HAF), which were GST-P positive. The GST-P incidence was 0.51 $\pm$ 0.3 and the severity was minimal, with an average size of ~204µ. At 16 weeks, the incidence and severity of HAF was increased in a dose-related manner in all methyl eugenol groups. The incidence of GST-P positive HAF value of 1.26 $\pm$ 0.24 in the high dose group, was significantly ($p< 0.01$) increased compared to low and mid dose groups. Also, the severity in the high dose group increased from minimal to mild, and the average size from ~204 to ~ 918µ. This phenomenon is in accordance with the previously published literature (Abdo *et al.*, 2001; Herbert and Abdo, 2003). Based on these data and other data from this laboratory, the following conclusions can be made:

- There is a clear dose response for the development of GST-P foci and positive hepatocellular altered foci (HAF)/cm^2 from the liver tissue of rats treated for 8 and 16 weeks. The induction of HAF reflects development of promotable preneoplastic lesions, which, it is anticipated, will be promoted to tumors with Phenobarbital (PB) in the promotion phase (now on week 35).
- Compared to an activation-dependent DNA-reactive hepatocellular carcinogen, 2-acetylaminofluorene (AAF), at both carcinogenic and non-carcinogenic doses (with PB promotion) in male F-344 rats, the GST-P positive HAF values at 16 weeks in the methyl eugenol HD group at a cumulative dose of 12000 mg/kg methyl eugenol were 15 times lower with methyl eugenol compared to AAF given at a cumulative dose of 94 mg/kg at 12 weeks (Williams *et al.*, 1998; 2004).
- Thus, the initiating activity of methyl eugenol can be considered weak.

Application of the Margin of Exposure (MoE) approach to Methyl Eugenol

Smith et al., recently applied the margin of exposure (MoE) approach to substances in food that are genotoxic and carcinogenic using methyl eugenol as an example. The MoE approach relies on the comparison of benchmark dose levels (BMDL) for carcinogenicity with different estimates of human intake. Based on all models applied, the BMD for a 10% incidence

of male rat liver adenomas and carcinomas (combined) is 7.9 mg/kg-bw/day. Using the estimated human daily intakes of 10 µg/kg-bw/day (Smith *et al*., 2002) up to a maximum theoretical intake of 66 µg/kg-bw/day for high consumers (EFSA, 2004), MoE of 800 and 100 were determined. The authors noted the MoE values were extremely low and further elaborated on the caveats influencing MoE results, specifically metabolic shifting, possible matrix effects from intake of methyl eugenol and related alkoxy-substituted allylbenzenes and the "nature and implications of the dose response curve in rats a low levels of exposure" (Smith et al., 2010). The authors also recommended additional studies were needed and noted the complexity in the reflection of these caveats in a numerical MoE (Smith et al., 2010).

Conclusions

In this review, a mechanism-based approach is used to present an example illustrating how minor structural changes may change metabolic pathways for bioactivation and detoxification in such a way that overall toxicity is significantly affected. This was demonstrated by comparing the metabolic fate and toxicologic sequelae of a series of naturally occurring food-borne substances that vary in structure only in the position of the double bond on the alkenyl side chain or the presence of a *para*-hydroxy or *para*-methoxy functional group. The impact of subtle structural changes on the mode of action for the four types of substances demonstrates that a mode-of action-based approach, in which the relationship between chemical structure and ADME characteristics leading to either preferential detoxification or formation of a bioactivated metabolite, can be used to interpret the potential pathologic, genotoxic and carcinogenic effects of these related food-borne natural compounds. It is concluded that isomerization of the double bond in the allyl side chain or the presence of a free phenolic hydroxyl moiety shifts the metabolism in favor of detoxification and eliminates the possibilities for formation of a genotoxic carcinogenic metabolite. This explains the remarkable differences in the toxicity of these related hydroxy- and alkoxy- substituted allyl- and propenyl-benzenes, and illustrates how subtle structural changes can have significant effects on toxicity.

In a second study of methyl eugenol, the initiating of hepatic tumor formation was evaluated in an 8 and 16-week study using phenobarbital as a tumor promoter. Although in the LD group, there was evidence of DNA adducts, cell proliferation (RF), and hepatocellular altered foci (HAF), there

was no evidence of initiation to hepatocellular adenomas. This lack of tumorigenic response occurred at a daily dose of 62 mg/kg bw/dose or a cumulative dose of 3000 mg/kg. At this level, there was no increase in hepatocellular adenomas with or without PB promotion. Thus, the dose level of 62 mg/kg bw/dose, represents a NOAEL dose for neoplasia, which is not capable of producing promotable hepatocellular neoplastic induction, under these experimental conditions, in spite of the presence of DNA adducts, increased RF and HAF. Identification of a lower dose that does not induce these effects would contribute to the risk assessment of methyl eugenol. In conclusion, methyl eugenol exhibits a low carcinogenic potential compared to that of 2-acetylaminofluorene and present no significant risk to humans under current conditions of use.

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