

**Use of physiologically based kinetic modelling
facilitated read-across in risk assessment of botanical
food-borne alkenylbenzenes**

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Thesis

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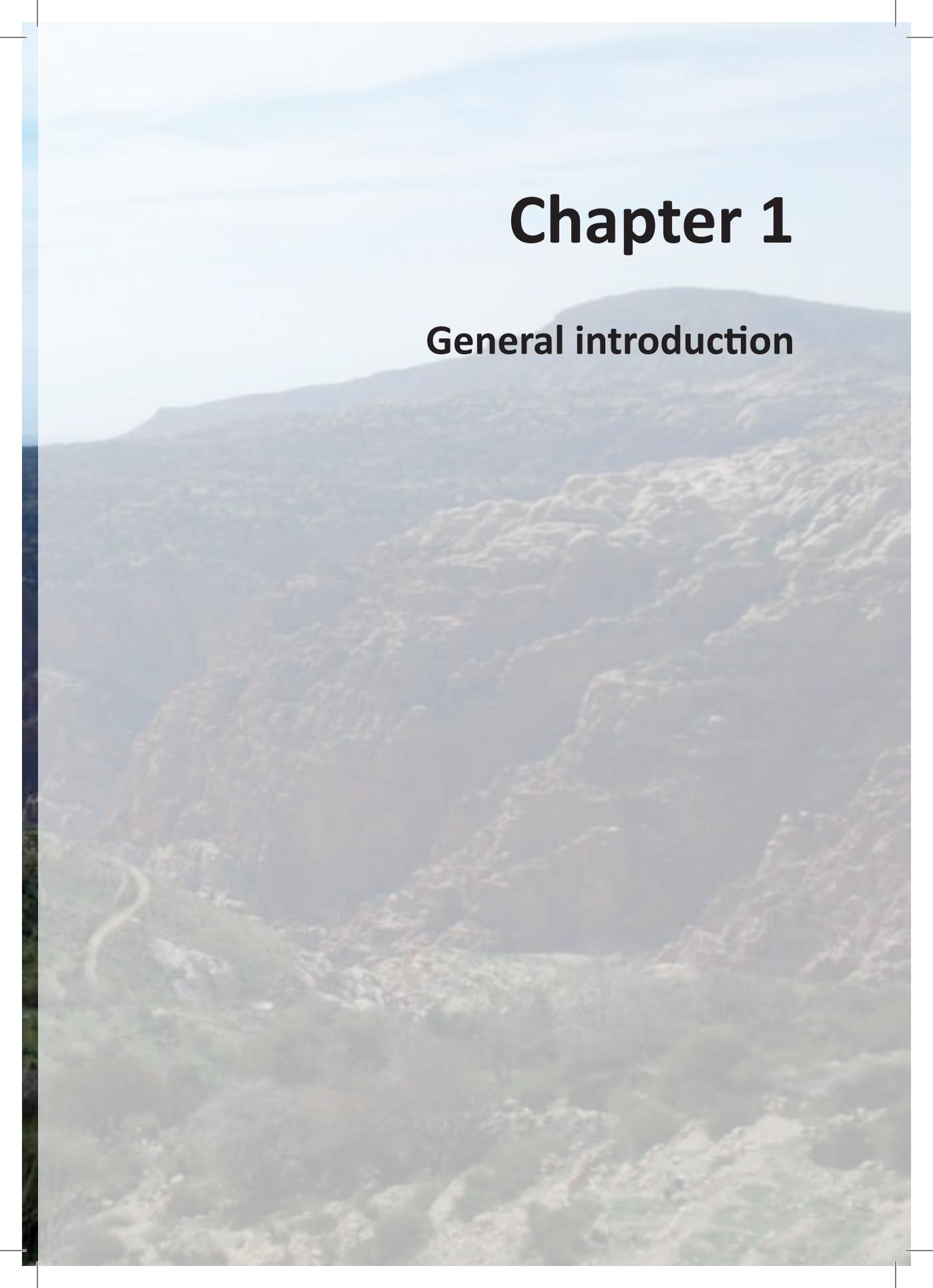
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Chapter 1

General introduction



Description and aim of the thesis

Botanicals and botanical preparations have become widely available in the form of food supplements and other food preparations. In Europe the safety of botanicals and their derived food products has to comply with the general requirements set out in regulation (EC) No 178/2002. Risk assessment procedures which consist of hazard identification, hazard characterization, exposure assessment, and risk characterization, evaluating the consequences of exposure to different classes of chemicals in food (e.g. additives, contaminants, pesticide residues, veterinary drug residues and natural toxicants) have been harmonized (Barlow et al. 2006; EFSA 2009a; WHO 2015). So far these regulatory procedures did not focus on botanicals and botanical preparations. In 2009 the European Food Safety Authority (EFSA) published a toolkit to help assess the safety of botanicals and derived preparations. A compendium of botanicals that have been reported to contain substances that may be of health concern when used in food or food supplements was part of that tool (EFSA 2012a). Also guidelines for risk assessment of botanicals and botanical preparations were provided (EFSA 2009a) indicating that a risk assessment should include the definition of the botanical species including the plant and preparation(s) of interest, environmental conditions of growing, part(s) of the botanical used, the manufacturing processes used for making the botanical preparation, evaluation of the compositional, toxicological and exposure data, definition of the biologically active substances and their levels, and the history of use. In spite of these guidelines, at the present state-of-the-art the safety and risks of botanicals and botanical preparations are generally not assessed before they enter the market.

An important aim of the present thesis was to perform risk assessment of selected botanicals and their derived preparations focusing especially on samples likely to contain alkenylbenzenes,

including myristicin, apiol, safrole, methyleugenol, elemicin, and estragole (Figure 1), to give a better understanding of when risk management actions would be needed for botanicals and derived preparations containing these ingredients. Samples containing alkenylbenzenes may be of concern because these compounds may be genotoxic and carcinogenic displaying similar characteristics regarding mode of action (MOA) and tumor formation (Phillips et al. 1981; Miller et al. 1983; Randerath et al. 1984; Wiseman et al. 1985; NTP 2000; Zhou et al. 2007; Kobets et al. 2016). In the present thesis a MOA based risk assessment of especially two of the alkenylbenzenes, myristicin and apiol, for which tumor data are not available, was performed. This was done using physiologically based kinetic (PBK) modelling and read-across from safrole and the margin of exposure (MOE) approach. Botanical preparations included in the risk assessments performed in the present thesis were basil-containing pesto and nutmeg-based plant food supplements (PFS). Given that several of the preparations analyzed contained several alkenylbenzenes a risk assessment of combined exposure to these alkenylbenzenes was included as well.

Physicochemical properties of the structurally related alkenylbenzenes

The alkenylbenzenes of interest are allylbenzenes with alkoxy substitution at the *para* position. The alkenylbenzenes safrole (3,4-methylenedioxyallylbenzene), myristicin (5-methoxy-3,4-methylenedioxyallylbenzene) and apiol (2,5-dimethoxy-3,4-methylenedioxyallylbenzene) have a 3,4-methylenedioxy substituent (Figure 1). The other compounds, estragole (4-methoxyallylbenzene), methyleugenol (3,4-dimethoxyallylbenzene) and elemicin (3,4,5-trimethoxyallylbenzene) do not contain a 3,4-methylenedioxy substituent (Figure 1). All molecules, except safrole, contain one or more methoxy groups.

The aforementioned alkenylbenzenes are colorless or slightly yellow oily liquids, with the characteristic odor of the plants from which they can be extracted. They are practically insoluble in water with a maximum solubility of 500 mg/l, and have octanol/water partition coefficients (log K_{ow} values) ranging from 3.0 to 3.5. They are miscible with most organic solvents, and generally denser than water (EPA 1986b). Because these alkenylbenzenes are structurally related, they may share similar metabolism, MOA and biological effects (JECFA 2009; van den Berg et al. 2012). This similarity provides the basis for the MOA based read-across and risk assessments of the present thesis.

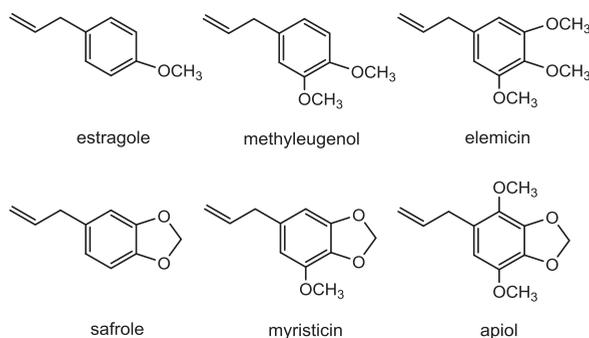


Figure 1. The structural formulas of the alkenylbenzenes estragole, methyleugenol, elemicin, safrole, myristicin and apiol.

Natural occurrence and estimated daily intake (EDI) of alkenylbenzenes from different sources

The alkenylbenzenes are naturally occurring in a variety of botanicals including anise, star anise, fennel, sweet fennel and parsley that belong to the plant family *Umbelliferae*, nutmeg and mace that belong to the plant family *Myristicaceae*, sweet and exotic basil that belong to the family *Labiatae*, and tarragon that belongs to the plant family *Compositae*. The intake of the

alkenylbenzenes occurs from consumption of spices and herb and spice oils. The levels of alkenylbenzenes in spices vary significantly depending on exact growing region, plant maturity at harvest, harvesting techniques, storage conditions and processing methods (e.g. extraction, drying). Nutmeg is the main source of exposure to myristicin, safrole, and elemicin, with an average content of 5292, 2099, and 1395 mg/kg respectively, as based on data from a variety of sources, including industry data and published reports (JECFA 2009; van den Berg et al. 2012). Exposure to estragole and methyleugenol occurs mainly by consumption of basil, with an average content of 1162 mg/kg for estragole and 62.8 mg/kg for methyleugenol, depending on harvest and storage time, and plant height (maturity) (Miele et al. 2001). Intake of apiole is predominantly from consumption of parsley with an average content of 8.7 mg/kg (JECFA 2009).

Plant Food Supplements (PFS) are concentrated sources of dietary ingredients derived from various plants and herbal extracts. Exposure to alkenylbenzenes through PFS is dependent on the level of the alkenylbenzenes in the PFS and amount and frequency of PFS intake. PFS made of essential oils were found to contain highly concentrated levels of alkenylbenzenes, and may result in high intakes (Archer 1988; van den Berg et al. 2011). General concerns related to the quality and safety of botanicals and botanical preparations such as PFS are often related to the presence of naturally occurring toxic compounds (EFSA 2004; JECFA 2009). Especially the use of highly concentrated plant material, which is usually marketed in the form of capsules or powder and supposed to supplement the normal diet, raises concerns.

Exposure to myristicin, safrole, estragole, and methyleugenol from food occurs mostly from basil, nutmeg, and parsley. The estimated daily intake (EDI) of these four alkenylbenzenes from the use of spices and spice oils ranges from 0.9 to 166 $\mu\text{g}/\text{kg}$ bw per day, with the EDI of safrole

being the lowest and that for estragole the highest (JECFA 2009). The dietary intakes of elemicin and apiole from all sources, with EDI values of 0.04 and 0.43 $\mu\text{g}/\text{kg}$ bw per day, respectively (JECFA 2009), were reported to make a minor contribution to the overall alkenylbenzene intake. With respect to alkenylbenzene exposure, pesto paste eaters are considered the highest exposed group, with 20 mg methyleugenol on average in each meal, making the daily consumption amount to 250 $\mu\text{g}/\text{kg}$ bw per day of methyleugenol for a 70 kg person (Miele et al. 2001). In 2001 The Scientific Committee on Food (SCF) concluded that the structural analogues, methyleugenol, estragole, and safrole are genotoxic and carcinogenic and indicated the need for a reduction in exposure and restrictions in the levels of use (SCF 2001a; SCF 2001b; SCF 2002). However, SCF or EFSA opinions on myristicin, apiole and elemicin are not available. Further investigation is needed to assess the potential risk to human health from low-level dietary exposure to alkenylbenzenes present in foods and essential oils (JECFA 2009). To this end, the EDI values in the present thesis will be calculated based on alkenylbenzene levels in samples containing alkenylbenzenes purchased from the market, taking into consideration the possible combined exposure to different alkenylbenzenes. Combined exposure can be evaluated by dose addition, which is considered appropriate for mixtures with components acting by a similar MOA, at a similar target organ, and causing a similar adverse effect i.e. liver carcinogenicity (EPA 1986a; EPA 1988; EPA 2000). This implies that the response to the mixture can be predicted by summing up the EDIs of the alkenylbenzenes assuming equal potency, or using their toxic equivalency factors (TEFs) relative to a reference alkenylbenzene. This requires definition of relative potency factors (RPFs) or toxic equivalency factors (TEFs) which were not available at the start of these studies.

Absorption, Distribution, Metabolism and Excretion (ADME) of alkenylbenzenes

Many studies on the fate and behavior of alkenylbenzenes were performed in *in vitro* models and in experimental animals, and results thus obtained were important in the interpretation of toxicity and extrapolation to humans (IPCS 1986a; Lipscomb and Ohanian 2007).

Toxicokinetic and metabolic experiments for alkenylbenzenes indicate that they undergo rapid and complete absorption following oral intake (Kamienski and Casida 1970; Fritsch et al. 1975; Fritsch et al. 1975; Benedetti et al. 1977; Sutton et al. 1985; Anthony et al. 1987; Lee et al. 1998).

The metabolites of elemicin, myristicin, and safrole have been identified in rat urine and the presence of some of them has been demonstrated in human urine of a nutmeg abuser (Beyer et al. 2006) (see also below). Figure 2 shows the proposed metabolic pathways of myristicin, which are similar to those for the structurally related alkenylbenzenes (Punt et al. 2008; JECFA 2009; Al-Subeihi et al. 2011; Martati et al. 2012; van den Berg et al. 2012). The primary biotransformation process of alkenylbenzenes is *O*-demethyl(en)ation that yields a phenolic derivative, that is mostly excreted as the sulfate or glucuronic acid conjugate (Benedetti et al. 1977; Zangouras et al. 1981; Sangster et al. 1983; Anthony et al. 1987; Lee et al. 1998; Beyer et al. 2006). Epoxidation of the double bond in the allyl side-chain is the alternative biotransformation pathway, yielding the 2',3'-epoxide. *In vivo*, the epoxide is rapidly detoxified by epoxide hydrolase or via glutathione conjugation and this prevents it from forming detectable levels of DNA adducts (Luo et al. 1992; Luo and Guenther 1995). Hydroxylation of the alkene side-chain to yield the 1'-hydroxy metabolite is considered the primary bioactivation pathway (Drinkwater et al. 1976; Benedetti et al. 1977; Zangouras et al. 1981; Miller et al. 1983). The 1'-hydroxymetabolite can be conjugated with either glucuronic acid or sulfate. The sulfate

conjugate is an unstable sulfate ester, and is considered to be the proximate hepatocarcinogenic agent (Smith et al. 2002) since it hydrolyses to form a reactive electrophilic intermediate, which binds to DNA. Rodent studies have shown that the formation of DNA adducts in the liver of exposed rodents is dose dependent (Drinkwater et al. 1976; Swanson et al. 1981; Miller et al. 1982; Boberg et al. 1983; Miller et al. 1983; Caldwell et al. 1992; Chan and Caldwell 1992; Hasheminejad and Caldwell 1994; Gardner et al. 1995; Daimon et al. 1998). The relative contribution of the different biotransformation routes of the alkenylbenzenes was also shown to be dose dependent and to show species and sex differences (Table 1). From all these data it is concluded that at low doses in humans, mice and rats, significant amounts of alkenylbenzenes are *O*-dealkylated. When dose levels increase, the relative amount of 1'-hydroxylation increases. In all species bioactivation to 1'-hydroxy occurs, but there may be species differences and/or differences between the alkenylbenzenes: so quantifying it for each congener is essential to obtain insight in relative bioactivation of the different alkenylbenzenes. The overview presented in table 1 also reveals that detection of a 1'-hydroxy alkenylbenzene in urine reflects its glucuronidation and thus its detoxification rather than its bioactivation by sulfation. This implies that relative differences in bioactivation and detoxification for each specific congener in rat and human cannot be derived from urinary metabolite patterns, and remain to be established before you can do read-across for risk assessment. The observation that the 1'-hydroxy metabolites of myristicin, safrole and elemicin are present in the urine of rats administered pure alkenylbenzenes at 100 mg/kg bw via oral intubation but absent from the urine of rodents or humans consuming nutmeg (50 times lower amount) may be due to dose or matrix effects.

Table 1. Overview of biotransformation studies on alkenylbenzenes.

Compound	Administered dose of pure compound or botanical (mg/kg bw)	Administration route	Species/ sample type/ sampling time (hr)	Result	Reference
Safrole	1.66 mg of [$1^3\text{-}^{14}\text{C}$]	Oral	Human/ urine/24	<i>O</i> -demethylation (major metabolite) (65% of the dose), and no $1^3\text{-}^{14}\text{C}$ hydroxy metabolite	(Benedetti et al. 1977)
	0.90-600 of [$1^3\text{-}^{14}\text{C}$]		Rat/ urine/ 48	<i>O</i> -demethylation (major metabolite) at lower dose (up to 60 mg/kg bw), relative amount decreased when dose increased (60 to 600 mg/kg bw). $1^3\text{-}^{14}\text{C}$ hydroxy metabolite was detected.	
Estragole	0.05 to 1000	Rats orally, mice intraperitoneally	Mice and rats / urine, faeces and exhaled air / 24	<i>O</i> -demethylation relative amount decreased (40% to 20% in both mice and rats of the dose) while $1^3\text{-}^{14}\text{C}$ -hydroxylation increased about 200 times. Dose-dependent increase in excreted glucuronic acid conjugate of the $1^3\text{-}^{14}\text{C}$ hydroxy metabolite.	(Zangouras et al. 1981)
Estragole	0.05- 1000 of [$1^3\text{-}^{14}\text{C}$]	Rats orally, mice intraperitoneally	Mice and rats / urine, faeces and exhaled air / 24	At low dose <i>in vivo</i> , <i>O</i> -demethylation major metabolic, at high dose $1^3\text{-}^{14}\text{C}$ hydroxylation and epoxidation of the allyl side-chain compete to a larger extent. The relative extent of $1^3\text{-}^{14}\text{C}$ -hydroxylation of estragole was slightly higher in rats after oral dosing than in mice after intraperitoneal administration and increased with increasing dose in both species.	(Anthony et al. 1987)
Safrole or myristicin	0.6 of [$1^3\text{-}^{14}\text{C}$] of each	Oral	Male Swiss- Webster mice/ urine, faeces and exhaled air/ 48	61% and 73% of dose via <i>O</i> -demethylation, 23% and 3% of dose excreted in urine, and 15% and 3% of the dose excreted in faeces for safrole and myristicin respectively.	(Kamienksi and Casida 1970)

Table 1. (Continued) overview of biotransformation studies on alkenylbenzenes.

Compound	Administered dose of pure compound or botanical (mg/kg bw)	Administration route	Species/ sample type/ sampling time (hr)	Result	Reference
Myristicin	100	Oral	Specific pathogen-free male Sprague-Dawley rats	<i>O</i> -demethylation and 1'-hydroxymyristicin were primary metabolites as glucuronic acid conjugates	(Lee et al. 1998)
Estragole	0.0015 [methoxy- ¹⁴ C]	Oral	Male human /urine/ 48	<i>O</i> -demethylation major (72% of the dose)	(Sangster et al. 1987)
Estragole	100	Oral and intraperitoneal	Rat/ urine/ 48	<i>O</i> -demethylation major (39% oral, 46% intraperitoneal of the dose), 1'-hydroxymetabolite (5-10% of the dose).	(Solheim and Scheline 1973)
Safrole	300	Oral	Rat/ urine/ 11-weeks	1'-hydroxysafrole (7%)	(Borchert et al. 1973)
		Intraperitoneal	Male guinea-pigs or hamsters, male and female mice/ urine/ 11-weeks	1'-hydroxysafrole 2.3 % and 3.5% of the dose in male guinea-pigs or hamsters, up to 33% of the dose for male mice, and approximately 17% of the dose for female mice	

Table 1. (Continued) overview of biotransformation studies on alkenylbenzenes.

Compound	Administered dose of pure compound or botanical (mg/kg bw)	Administration route	Species/ sample type/ sampling time (hr)	Result	Reference
Estragole and saffrole	Estragole 274, saffrole 300	Oral	21-day-old mice/ urine/ 24	1'-hydroxymetabolite (23% of the dose for estragole and 12% of the dose for saffrole)	(Drinkwater et al. 1976)
	saffrole 300	Intraperitoneal	Adult male mice/ urine/ 24	1'-hydroxymetabolite 46% of the dose	
Elemicin, myristicin and saffrole	100	Oral	Male Wistar rat/ urine/ 24	For rats given ground nutmeg, no 1'-hydroxylation metabolite was detected.	(Beyer et al. 2006)
	500 of ground nutmeg (approx. 1-2 myristicin)	Oral	Male Wistar rat	For both rats and humans exposed to nutmeg, the corresponding <i>O</i> -demethylmetabolite of saffrole or myristicin was the predominant	
	3.5, 2.4, 0.3 of elemicin, myristicin, and saffrole	Oral	Human	metabolite, exceeding other metabolites by at least a factor of 10. The side-chain demethylation products were the major metabolites of elemicin	

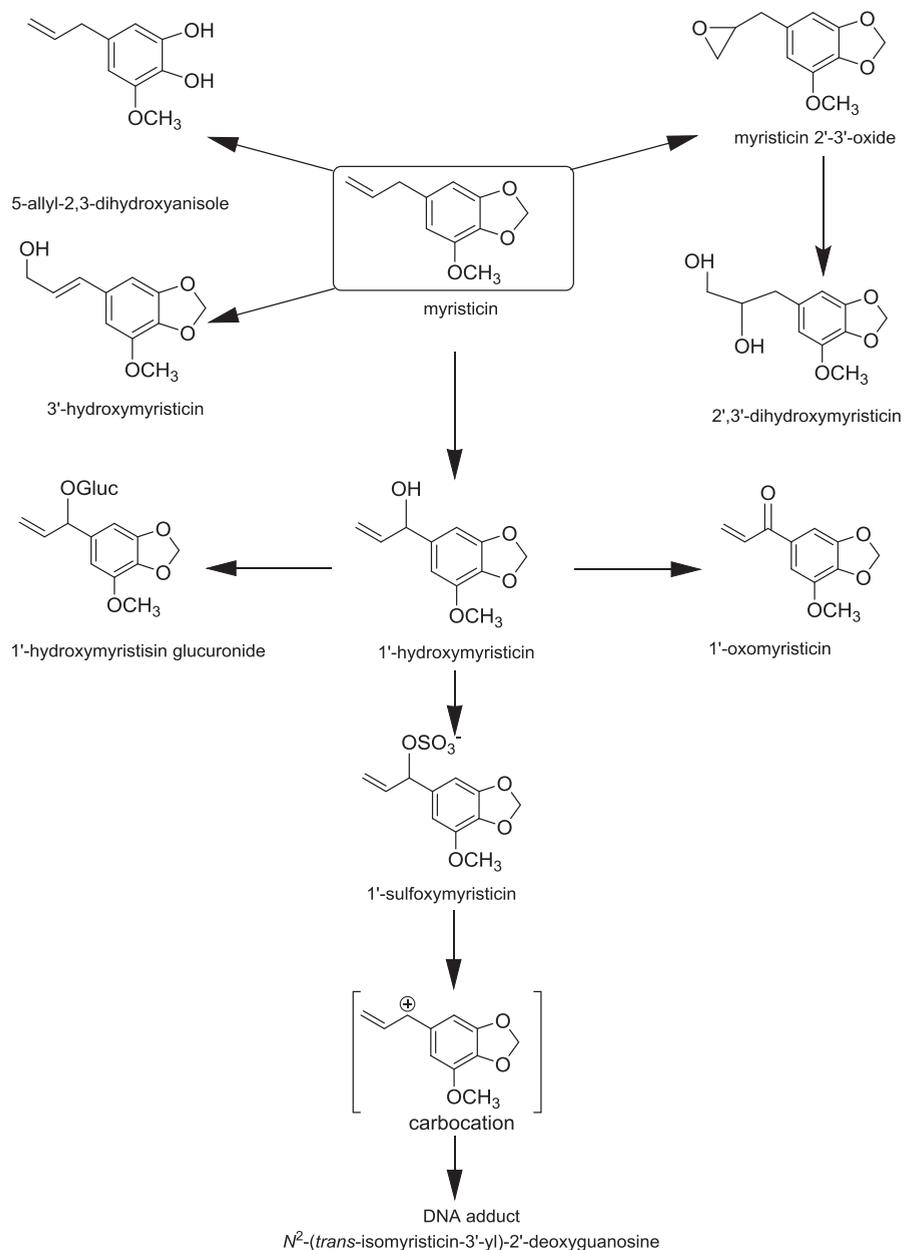


Figure 2. Proposed metabolic pathways of the alkenylbenzene myristicin (Kamienski and Casida 1970; Sangster et al. 1987; Lee et al. 1998; Beyer et al. 2006).

Carcinogenicity and MOA

The adverse critical effect of the alkenylbenzenes is the formation of liver tumors, and for some of the alkenylbenzenes long term carcinogenicity studies in rodents are actually available (Hagan et al. 1967; Borchert et al. 1973; Lipsky et al. 1981a; Lipsky et al. 1981b; Lipsky et al. 1981c; Miller et al. 1983; NTP 2000) (Table 2). The purpose of testing chemicals for carcinogenicity in a chronic lifetime exposure scenario with experimental animals is to identify potential cancer hazards for humans. Positive findings require careful interpretation in relation to MOA, possible interspecies differences in background incidence and in response and with respect to high dose to low dose extrapolation (WHO 2009b). Virtually all chemicals associated with cancer in humans have been found to increase the incidence of neoplasms in experimental animals albeit not causing necessarily the same type of tumor (McGregor and Anderson 1999). For alkylating compounds, genetic changes arise from the reactivity of the carcinogen with DNA, because the reactive proximate carcinogen may operate as an electrophilic reactant covalently binding to DNA as a fundamental part of the MOA underlying induction of carcinogenesis. Carcinogens that act through such genotoxic mechanisms are usually multi-organ and trans-species carcinogens that can be active with a single dose and are effective at low exposures (Vaino et al. 1992; Williams 1992).

³²P-postlabeling indicated that estragole, safrole, methyleugenol, myristicin, elemicin, and apiol have the ability to produce DNA adducts in adult mouse liver upon intraperitoneal injections. Estragole, methyleugenol and safrole showed the highest activity of the alkenylbenzenes tested (Randerath et al. 1984). Similar results were shown by Phillips et al. 1984, who reported that in neonatal mice upon intraperitoneal injections the ability to form DNA adducts was in the following order; methyleugenol > estragole > safrole > myristicin > apiol (Phillips et al. 1984).

Using human HepG2 cells exposed *in vitro*, the level of DNA adduct formation by estragole, methyleugenol and safrole was the highest at low concentration (50 $\mu\text{mol/l}$), while at higher concentration (450 $\mu\text{mol/l}$), the level of adduct formation of myristicin was as high as that of safrole and methyleugenol (Zhou et al. 2007).

Long-term carcinogenicity data from studies with rodents given high dose levels showed increased incidences of hepatocellular carcinoma especially with safrole, estragole and methyleugenol (Table 2). These studies indicate that the structurally related alkenylbenzenes are compounds that are genotoxic and carcinogenic, with relative difference in potency. Similar long term carcinogenicity studies for myristicin, elemicin and apiole are not available.

Risk assessment approach

In the present thesis risk assessment of consumption of the alkenylbenzene-containing samples was performed using the MOE approach. The MOE approach is the approach recommended for risk assessment of compounds that are both genotoxic and carcinogenic (EFSA 2005). The MOE is a dimensionless ratio comparing a standardized reference point derived, by mathematical modelling, from the animal data within the observed range of experimental data, with the EDI of the compound of interest resulting from the use of food containing that compound. The EFSA Scientific Committee considered that an MOE of 10000 or more, based on animal cancer bioassay data, “*would be of low concern from a public health point of view and might reasonably be considered as a low priority for risk management actions*” (EFSA 2005). The EFSA recommends the use of the BMDL_{10} as a reference point for MOE calculations, which is the lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence above background level (EFSA 2009a; EFSA 2009b). In previous studies, the BMDL_{10} values for

safrole, estragole, and methyleugenol could be derived from *in vivo* tumor data (van den Berg et al. 2011). Table 3 presents an overview of these BMDL₁₀ values. However, not for all alkenylbenzenes tumor data enabling definition of a BMDL₁₀ for risk assessment are available. Such data are missing for elemicin, myristicin and apiol. Previously, a BMDL₁₀ value for elemicin was derived by read-across from methyleugenol and estragole (EFSA 2005; van den Berg et al. 2012) (Table 3). In the present thesis a similar approach with read-across from safrole was undertaken for myristicin and apiol.

Van den Berg et al. (2011) investigated the alkenylbenzene content in 28 PFS samples containing botanicals including basil, fennel, saffron, nutmeg, cinnamon, and calamus (van den Berg et al. 2011), and performed a risk assessment using the MOE approach. The results obtained revealed that out of 28 PFS samples, 25 samples contained alkenylbenzenes at levels that would result in EDIs that would give rise to MOE values below 10000, indicating a priority for risk management. Among these 25 PFS samples, there were 4 nutmeg-based samples shown to contain methyleugenol and safrole at levels that would result in EDI values that gave rise to MOE values < 10000 (van den Berg et al. 2011). In addition to safrole and methyleugenol, nutmeg may also contain myristicin and elemicin (JECFA 2009).

Table 2. Summary of the results of long-term carcinogenicity studies of alkenylbenzenes

Compound	Species; sex	Administration route	No. of test animals per group)	Dose administered (mg/kg bw)	Duration	Tumor incidence (%)	Reference
Estragole	Pre-weanling male CD-1 mice	Gavage	50	370	14 months	73	(Miller et al. 1983)
	Pre-weanling female CD-1 mice					9	
	CD-1 female mice, 8 weeks old	Grain diets	50	2300 4600	12 months	56	
	Pre-weanling CD-1 male mice					70	
Methyl Eugenol	pre-weanling B6C3F1 male mice	Intraperitoneal injection	50	31.8	12 months	65	(Miller et al. 1983)
	Pre-weanling B6C3F1 male mice	Intraperitoneal injection	50	42.4	18 months	83	
	Pre-weanling B6C3F1 male mice	Intraperitoneal injection	58	37	18 months	97	
	B6C3F1 female mice	Gavage	50	75 150	2 years	6 28 50	
Safrole	Pre-weanling CD-1 male mice	Gavage	100	400	14 months	61	(Miller et al. 1983)
	Pre-weanling CD-1 female mice					13	
	Pre-weanling male CD-1 mice	Intraperitoneal injection	50	81	12 months	67	
	CD-1 female mice, 8 weeks old	Grain diets	50	2300 4600	19 months	68 69	

Table 3. BMDL₁₀ values of alkenylbenzenes as derived from *in vivo* tumor data for safrole, estragole, and methyleugenol, or obtained by read-across from methyleugenol and estragole for elemicin. NA, Not Available.

Compound	BMDL ₁₀ (mg/kg bw)	Reference
Methyleugenol	15.3-34.0	(van den Berg et al. 2011)
Safrole	1.9-5.1	
Estragole	3.3-6.5	
Elemicin	30.6-68.0	(van den Berg et al. 2012)
Myristicin	NA	NA
Apiol	NA	NA

The aim of the present study was to develop a MOA and PBK modeling based approach for read-across in risk assessment from safrole, for which *in vivo* toxicity studies are available to myristicin and apiol for which tumor data are not reported, thus contributing to alternatives in animal testing, and to use the outcomes for risk assessment for the consumption of PFS and other botanical products containing myristicin and structurally related alkenylbenzenes considering individual and combined exposure. The next section illustrates in some more detail how PBK modeling can be used to estimate BMDL₁₀ values for alkenylbenzenes for which no tumor data are available.

PBK modeling

PBK models are developed in order to study effects related to interspecies, intraspecies, high to low dose, route to route, and exposure scenario extrapolations using *in vitro* and *in vivo* kinetic data. PBK models, in general, are developed to integrate diverse sets of kinetics data, and predict tissue dosimetry for situations relevant to human dietary levels.

A PBK model is represented by a set of mathematical equations that together describe the process of a substance entering the body (absorption), dispersing throughout the fluids and tissues of the body (distribution), the biotransformation to its metabolites (metabolism), and the elimination from the body (excretion), having all the compartments mutually connected through the systemic circulation. Steps to build a PBK model are as follows (Rietjens et al. 2011); First define the structure of the model including organs and tissues that are of importance for the kinetic processes and the toxicity of the compound of interest. Second, mathematically describe the relevant processes, using equations for absorption, distribution, excretion and metabolism the latter including separate Michaelis–Menten equations for each metabolic conversion, one for each important metabolic route. Third, define the values of the model parameters needed to develop the PBK model for a specific compound. Fourth, using software, e.g. Berkeley Madonna, solve the mass-balance differential equations and predict the levels of the parent compound and its relevant metabolites at certain dose levels in the relevant compartments of the body in time. Fifth, evaluate the model against experimental *in vivo* data to support the validity of the model and thus its subsequent predictions. In a final step the model can be used to make predictions. The parameters needed for the model are physiological parameters (cardiac output, tissue blood flow rate, tissue volumes), physicochemical parameters (partition coefficients), kinetic and biochemical parameters (describing metabolic processes, rates of absorption, biotransformation). These parameters can be obtained from literature, experimentally (*in vitro* or *in vivo*), and/or by using *in silico* tools such as for example Quantitative Structure Activity Relationships (QSAR). A quantitative evaluation of how input parameters influence the model output of relevance to the risk assessment can be provided via a sensitivity analysis (Iman and Helton 1988; Farrar et al. 1989; Krewski et al. 1995; Campolongo and Saltelli 1997; Nestorov 2001; Gueorguieva et al. 2006; Chiu et al. 2007; Loizou et al. 2008). In a sensitivity analysis one input parameter

is changed (for example by 5 %) while the other parameters are kept at their initial value and it is estimated how the prediction is affected by that change. Sensitivity analysis results are presented as high (absolute value of the sensitivity coefficient greater than or equal to 0.5), medium (absolute value greater than or equal to 0.2 but less than 0.5) or low (absolute value of the sensitivity coefficient greater than or equal to 0.1 but less than 0.2); parameters with a sensitivity coefficient less than 0.1 are generally not listed (Kohn 1995; IPCS 2010).

Objectives and outline of the thesis

The aim of the present PhD project was to develop a MOA and PBK modeling based approach for read-across in risk assessment from safrole for which *in vivo* toxicity studies are available to myristicin and apiol for which tumor data are not reported, and to use the outcomes for risk assessment for the consumption of PFS and other botanical products containing myristicin and structurally related alkenylbenzenes considering individual and combined exposure.

The introduction chapter (this chapter) starts with a short background and definition of the aim of the thesis, followed by a description of the physicochemical properties, natural occurrence and EDI of the alkenylbenzenes, as well as of their ADME characteristics, carcinogenicity and MOA, the relevant risk assessment approach, and finally PBK modelling. Chapter 2 of the thesis describes the development of a PBK model for myristicin in male rat and human, enabling the prediction of dose-dependent effects in bioactivation and detoxification of myristicin. This allows comparison of the PBK model-based prediction of bioactivation of myristicin to the PBK model-based predictions for bioactivation of the structurally related compound safrole, enabling estimation of a BMDL₁₀ for myristicin from read-across to the BMDL₁₀ available for safrole, thereby facilitating risk assessment of current dietary exposure to myristicin.

Chapter 3 describes PBK models for the alkenylbenzene apiol in rat and human, based on the PBK models previously developed for the structurally related alkenylbenzenes safrole. Using the newly developed models, the level of metabolic activation of apiol in rat and human was predicted to obtain insight in species differences in the bioactivation of apiol and read-across to the related alkenylbenzene safrole. The models were also used for read-across from safrole to apiol and definition of a BMDL₁₀ for apiol.

Chapter 4 evaluates the risk of exposure to methyleugenol and related alkenylbenzenes through consumption of pesto sauce. To this end the levels of methyleugenol and other alkenylbenzenes in basil-containing sauce of pesto were quantified enabling an associated risk assessment based on the MOE approach, taking into consideration the possible combined exposure to different alkenylbenzenes and the BMDL₁₀ values as defined in literature (Table 3) and in chapter 2 and 3 of the thesis.

Chapter 5 evaluates the risk of exposure to myristicin and related alkenylbenzenes through human exposure to nutmeg-based PFS based on the MOE approach. Chemical analyses of various PFS samples were performed and the amount of alkenylbenzenes that would be consumed through consumption of these nutmeg-based PFS were quantified. MOE values were calculated for the individual alkenylbenzenes as well as taking into account the presence of more than one alkenylbenzenes within selected PFS samples.

Chapter 6 summarizes the results obtained in the thesis, compiles the overall discussion and presents the future perspectives that follow from the results obtained.

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Chapter 2

Physiologically based kinetic modeling of the bioactivation of myristicin

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ABSTRACT

The present study describes physiologically based kinetic (PBK) models for the alkenylbenzene myristicin that were developed by extension of the PBK models for the structurally related alkenylbenzene safrole in rat and human. The newly developed myristicin models revealed that the formation of the proximate carcinogenic metabolite 1'-hydroxymyristicin in liver is at most 1.8-fold higher in rat than in human and limited for the ultimate carcinogenic metabolite 1'-sulfoxymyristicin to (2.8–4.0)-fold higher in human. In addition, a comparison was made between the relative importance of bioactivation for myristicin and safrole. Model predictions indicate that for these related compounds, the formation of the 1'-sulfoxy metabolites in rat and human liver is comparable with a difference of < 2.2-fold over a wide dose range. The results from this PBK analysis support that risk assessment of myristicin may be based on the BMDL₁₀ derived for safrole of 1.9–5.1 mg/kg bw per day. Using an estimated daily intake of myristicin of 0.0019 mg/kg bw per day resulting from the use of herbs and spices, this results in MOE values for myristicin that amount to 1000–2700, indicating a priority for risk management. The results obtained illustrate that PBK modeling provides insight into possible species differences in the metabolic activation of myristicin. Moreover, they provide and contributing to alternatives in animal testing example of how PBK modeling can facilitate a read-across in risk assessment from a compound for which *in vivo* toxicity studies are available to a related compound for which tumor data are not reported, thus contributing to alternatives in animal testing.

INTRODUCTION

Myristicin (1-allyl-5-methoxy-3,4 methylene-dioxybenzene or methoxysafrole) is naturally occurring and present in several spices including nutmeg and mace of trees of *Myristica* species, principally *Myristica fragrans* Hout, and their essential oils (Forrest and Heacock 1972; Matthews et al. 1974; Sammy and Nawar 1968). There is a potential for human exposure to myristicin through foods, beverages, food supplements, and traditional medicines. Myristicin belongs to the group of alkenylbenzenes that contains structural analogues such as methyleugenol, estragole, elemicin, safrole, and apiol (Figure 1), compounds that are all naturally occurring in herbs and spices such as basil, nutmeg, and their essential oils (Barceloux 2009). Safrole, estragole, and methyleugenol have been shown to induce hepatic tumors in rats or mice upon chronic oral exposure to high doses and upon administration to male CD-1 mice during the preweaning period (Borchert et al. 1973; Drinkwater et al. 1976; Ioannides et al. 1981; Wislocki et al. 1977; Innes 1969; National Toxicology 2000). Tumors were found especially in the liver at frequencies that amounted to, for example, 2/50, 3/50, 14/50, and 25/50 at 0, 37, 75, and 150 mg methyleugenol/kg bw per day in male rats (National Toxicology 2000). A summary of further incidences of malignant tumor formation in mice and rats after administration of estragole, methyleugenol, or safrole can be found in our previous paper (van den Berg et al. 2011). However, myristicin is less well studied than its structurally related analogues, and only limited toxicological data are available. While *in vitro* genotoxicity studies indicate that myristicin is mutagenic and capable of inducing the formation of DNA adducts (EFSA 2012; Zhou et al. 2007b), no two-year carcinogenicity studies of myristicin in experimental animals are available hampering its risk assessment.

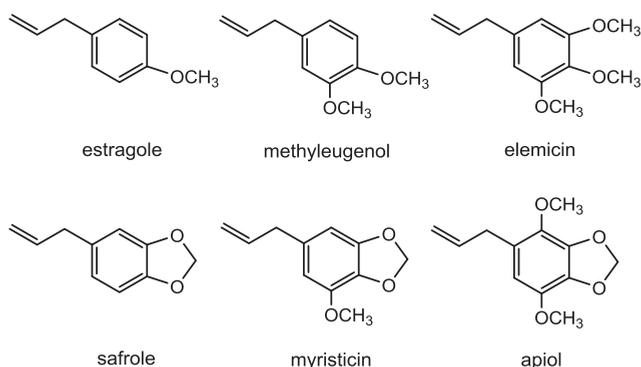


Figure 1. Structural formulas of the alkenylbenzenes, estragole, methyleugenol, elemicin, safrole, myristicin, and apiol.

Some short-term studies were conducted on the induction of hepatic tumors, in which male B6C3F1 mice were given myristicin during the preweaning period in two separate experiments. In the first experiment, myristicin was injected with a total dose of 3.75 μmol to 33 male B6C3F1 mice over a period of 22 days, and the total duration of the experiment was 12 months. In a second experiment, 45 male B6C3F1 mice were each injected with a total dose of 4.75 μmol myristicin over a period of 22 days, and the total duration of the experiment was 18 months. In these short-term exposure experiments, myristicin had no detectable activity for the initiation of hepatic tumors (Miller et al. 1983).

In agreement with hepatocarcinogenicity in mice (Miller et al. 1983), DNA adduct formation upon exposure to myristicin was generally lower and less persistent than DNA adduct formation upon exposure to methyleugenol and estragole and appeared to be about 2-fold lower than DNA adduct formation upon exposure to safrole, both in *in vitro* study (Zhou et al. 2007a) and in *in vivo* studies (Table 1) (Phillips et al. 1984; Randerath et al. 1984). N²-(trans-isomyristicin-3'-yl)-2'-deoxyguanosine was found to be the major myristicin DNA adduct formed when mice were

given cola drinks instead of water up to 8 weeks. In a parallel experiment, pregnant ICR mice were treated by gastric intubation with a single dose of 6 mg of myristicin, and the level of myristicin adducts in maternal and fetal liver was 68 and 63 % of the total adducts (Randerath et al. 1993). In freshly isolated rat hepatocytes in primary culture, safrole, estragole, and methyleugenol induced unscheduled DNA synthesis (UDS) and cytotoxicity at the concentrations of 10^{-6} to 10^{-3} M, (Howes et al. 1990) while myristicin showed cytotoxicity. In these studies, concentrations inducing UDS were generally close to or already at concentrations detecting cytotoxicity, hampering interpretation of the data. The data supporting a genotoxic mode of action for the tumor induction by the alkenylbenzenes rather come from studies reporting DNA adduct formation. In these studies, myristicin, as well as estragole, methyleugenol, and safrole, all showed positive results (Zhou et al. 2007a; Kobets et al. 2016; Phillips et al. 1984; Randerath et al. 1984). A schematic overview of the detoxification and bioactivation of myristicin that is similar to that of its structurally related compound safrole (Swanson et al. 1979; Borchert et al. 1973; Drinkwater et al. 1976) is shown in Figure 2. Epoxidation of the double bond in the allyl side chain yields the 2,3-epoxide. In *in vitro* experiments, the epoxide readily forms DNA adducts, but rapid detoxification by epoxide hydrolase and glutathione S-transferases (GSTs) prevents it from forming detectable levels of DNA adducts *in vivo* (Luo et al. 1992; Luo and Guenther 1996). The primary bioactivation pathway of myristicin is 1'-hydroxylation of the alkene side chain to yield the 1'-hydroxy metabolite which can be conjugated with either glucuronic acid representing a detoxification reaction or sulfate representing the ultimate bioactivation to 1'-sulfoxymyristicin (Drinkwater et al. 1976; Benedetti et al. 1977; Zangouras et al. 1981; Miller et al. 1983). The 1'-sulfoxy metabolite represents the ultimate carcinogenic metabolite (Wiseman et al. 1985, 1987;

Randerath et al. 1984; Phillips et al. 1984). 1'-Sulfoxy metabolites of alkenylbenzenes may react readily with DNA, RNA, and proteins but can also be detoxified through reaction with H₂O or conjugation with glutathione (Phillips et al. 1984; Miller et al. 1983; Fennell et al. 1984; Ishii et al. 2011). Therefore, only a fraction of the 1'-sulfoxy metabolite is expected to form DNA adducts (Rietjens et al. 2014).

Although myristicin is thus likely to also induce DNA adducts and liver tumors, its risk assessment at low realistic dietary intake levels is hampered by the fact that carcinogenicity studies are lacking (Hallstrom and Thuvander 1997). Therefore, the aim of the present study was to characterize the detoxification and bioactivation of myristicin and to develop a physiologically based kinetic (PBK) model to describe the ultimate formation of the 1'-sulfoxy metabolite in the liver of both rat and human and subsequently perform a read-across-based risk assessment using a similar PBK model previously described for safrole for which tumor data from long-term toxicity testing are available. Previously, using a similar approach and read-across from estragole and methyleugenol, the risk of exposure to elemicin for which also only limited *in vivo* rodent tumor data were available could be evaluated (van den Berg et al. 2012). Such a PBK modeling-based read-across illustrates a novel approach for animal-free risk assessment of a genotoxic carcinogen without the need for a long-term carcinogenicity study.

Table 1. Comparison of data on DNA adduct formation from *in vitro* and rodent studies using saffrole and myristicin.

Species	Sex	Route of admin.	Myristicin		Saffrole			DNA isolation and persistence study	Analysis technique of DNA adduct	Ref.
			Dose or conc.	RAL	Dose or conc.	RAL	Duration of Exposure			
Cultured Human HepG2 cells			50 ^a	5.67 ^c	50 ^a	9.12 ^c	The cells were treated with different doses, incubated, and then harvested after 720-hr		³² P-Postlabeling, studying DNA binding ability <i>in vitro</i>	(Zhou et al., 2007)
			150 ^a	16.27 ^b	150 ^a	27.66 ^c				
			450 ^a	55.10 ^b	450 ^a	33.93 ^c				
Mouse liver CD-1	Female	i.p	400 ^b	165 ^d	400 ^b	678 ^d	livers were collected 24-hr after treatment	after admin. Of dose 400 mg/kg.bw saffrole, livers were taken after 24-hr, 28, 58, 98, & 140 days after each treatment, after 140 days adduct was 21 pmol/mg DNA	³² P-Postlabeling, studying DNA binding ability <i>in vitro</i>	(Randerath et al., 1984)
			80 ^b	56 ^d	80 ^b	153 ^d				
New born mice B6C3F1	Male	i.p	36.5* ^a	7.8	30.8	17.5	22 days after birth (1, 8, 15, & 22 days of injection)	days 23, 29, & 43. Adducts were still available after 43 days	³² P-Postlabeling, studying DNA binding ability <i>in vivo</i>	(Phillips et al., 1984)

^afor dose in mg/kg bw, ^bfor concentration in μM , ^c for $\text{RAL} \times 10^6$, ^d for $\text{RAL} \times 10^7$

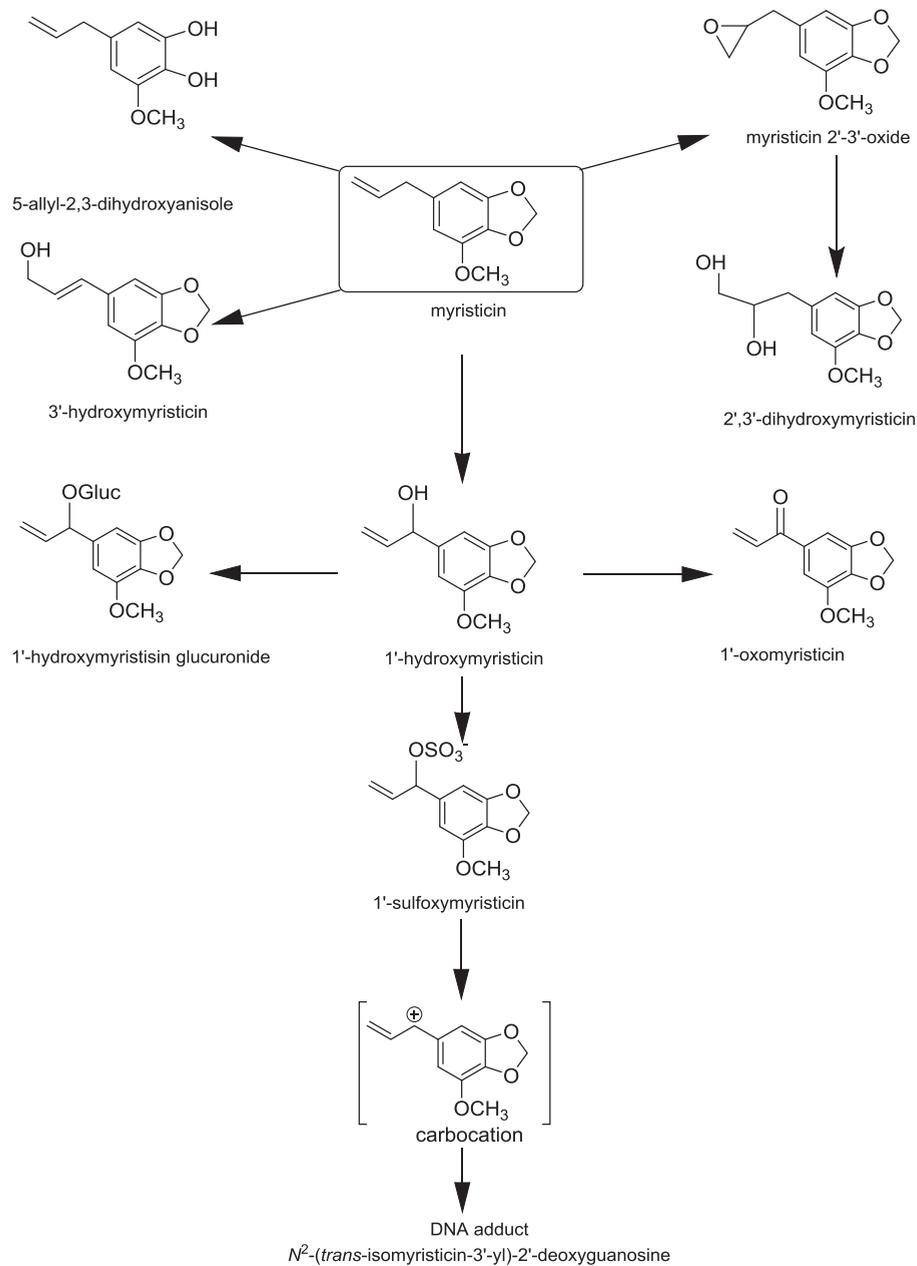


Figure 2. Proposed metabolic pathways of the alkenylbenzene myristicin.

MATERIAL AND METHODS

Chemicals

Myristicin, tris(hydroxymethyl)aminomethane, uridine 5'-diphosphoglucuronide acid (UDPGA), reduced L-glutathione (GSH), alamethicin (from *Trichoderma viride*), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), β -nicotinamide adenine dinucleotide (NAD⁺), and reduced β -nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Roche Diagnostics (Mannheim, Germany). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate trihydrate, acetic acid, and magnesium chloride were supplied by VWR International (Darmstadt, Germany). Acetonitrile (ACN) (UPLC/MS grade) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Trifluoroacetic acid TFA was obtained from Merck (Darmstadt, Germany). Pooled male rat liver microsomes and S9 from Sprague–Dawley and mixed gender pooled human liver microsomes and S9 were obtained from BD Gentest (Woburn, USA). Pooled male Sprague–Dawley rat lung, kidney, and small intestinal microsomes and pooled gender human lung, kidney, and intestinal microsomes were purchased from BioPredic International (Rennes, France).

Synthesis of 1'-hydroxymyristicin and 1'-oxomyristicin

The synthesis of 1'-hydroxymyristicin from myristicin was done as described previously by Jeurissen et al. (2004), and 1'-oxomyristicin was synthesized from 1'-hydroxymyristicin according to the method used for the synthesis of 1'-oxoestradiol from 1'-hydroxyestradiol (Wislocki et al. 1976).

Microsomal metabolism of myristicin

First, it was determined which organs are involved in the biotransformation of myristicin in rat and human. For this purpose, liver, kidney, lung, and small intestine microsomes from male Sprague–Dawley rat and pooled gender human were used. Incubations were performed by adding myristicin to incubation mixtures containing 1 mg/mL of the microsomal protein preparations and 3 mM NADPH in 0.2 M Tris–HCl (pH 7.4). After 1 min of pre-incubation at 37 °C, myristicin (final concentration, 1000 µM) was added from a 100 times concentrated stock solution in DMSO so that the final DMSO content was 1 % (v/v). Incubations were carried out for 30 min after which the reactions were terminated by adding 25 µl (0.25 times the incubation volume) of ice-cold ACN. Samples were centrifuged for 5 min at 13000×g, and the supernatant was stored at –20 °C until UPLC analysis. As metabolism of myristicin was observed only in incubations with liver microsomes for male rats and pooled gender human, the determination of kinetic constants for the formation of microsomal metabolites was performed only for the liver fractions. Incubations to determine kinetic constants were performed following the conditions described above using final concentrations of myristicin from 25 to 1000 µM for rat and human liver. In all incubations, the final concentration of DMSO, in which myristicin was dissolved, was kept at 1 % (v/v). The formation of different microsomal metabolites was linear with time and microsomal protein concentration under the conditions described. Blank incubations were performed in the absence of the cofactor NADPH. All incubations were performed in triplicate.

Glucuronidation of 1'-hydroxymyristicin to 1'-hydroxymyristicin glucuronide

The kinetic constants for the metabolic conversion of 1'-hydroxymyristicin to 1'-hydroxymyristicin glucuronide (HMG) by both male rat and human liver fractions were

determined as described previously for the related 1'-hydroxyalkenylbenzenes (Punt et al. 2008; van den Berg et al. 2012; Punt et al. 2009; Martati et al. 2011, 2012; Al-Subeihi et al. 2011, 2012). Briefly, incubations contained (final concentrations) 10 mM of UDPGA and 0.5 mg/ml of male Sprague–Dawley or pooled gender human S9 protein in 0.2 M Tris–HCl (pH 7.4) containing 10 mM of MgCl₂. To overcome enzyme latency and to obtain maximal glucuronidation activity, incubations containing S9 were pretreated on ice with 0.025 mg/ml alamethicin added from a 200 times concentrated stock dissolved in methanol (Fisher et al. 2000; Lin and Wong 2002). After 15 min of alamethicin treatment, samples were pre-incubated at 37 °C for 1 min, and reactions were subsequently started by adding 1'-hydroxymyristicin at final concentrations of 10 to 1200 µM. 1'-Hydroxymyristicin was added from 100 times concentrated stock solutions in DMSO. The reaction mixtures were incubated for 30 and 180 min for rat and human, respectively, and terminated by adding 25 µl (0.25 times the incubation volume) of ice-cold ACN. Blank incubations were carried out in the absence of the cofactor UDPGA. Experiments were performed in triplicate. The longer incubation time in human samples was required due to the lower rate of glucuronidation. The formation of 1'-hydroxymyristicin glucuronide was linear with time and the S9 protein concentration under the experimental conditions described. All samples were centrifuged for 5 min at 16000×g, and the supernatant was stored at –20 °C until ultra-performance liquid chromatography (UPLC) analysis.

Oxidation of 1'-hydroxymyristicin to 1'-oxomyristicin

The kinetic constants for the enzymatic conversion of 1'-hydroxymyristicin to 1'-oxomyristicin were determined using incubation mixtures containing (final concentrations) 3 mM NAD⁺, 2 mM GSH, and 1 mg/ml rat or human liver microsomes in 0.2 M Tris–HCl (pH 7.4). GSH was

added to the incubation mixtures to trap the reactive 1'-oxo metabolite formed after oxidation of 1'-hydroxymyristicin. The level of GSH in the incubations was optimized in a previous study to obtain maximum scavenging of 1'-oxoestragle (Punt et al. 2009). To this end, incubations were performed in the presence of increasing concentrations of GSH, ranging from 2 to 10 mM. At a concentration of 2 mM GSH maximum formation of GS-1'-oxoestragle was reached in the incubations, pointing at maximum scavenging of 1'-oxoestragle at this concentration (Punt et al. 2009). Kinetic constants for this reaction in rat and human liver were derived by performing incubations with NAD⁺ as a cofactor, given that in rat and human liver, NAD⁺ is mainly present in an oxidized form with levels of NAD⁺ being much higher than those of NADH. In a previous study, it was shown that the highest level of 1'-hydroxy alkenylbenzene oxidation occurs in incubations with microsomes in the presence of NAD⁺ as cofactor. In incubations with pooled human liver cytosol in the presence of NAD⁺ or NADP⁺, lower levels of oxidation were observed, indicating that the reaction is not primarily catalyzed by alcohol dehydrogenases (ADH) or other enzymes present in the cytosol (Punt et al. 2010). The enzyme responsible for the oxidation may be 17 β -hydroxysteroid dehydrogenase type 2 (17 β -HSD2) (Punt et al. 2010). Prior to the addition of 1'-hydroxymyristicin at final concentrations ranging between 10 and 4000 μ M to the incubation mixture from 100 times concentrated stock solutions in DMSO, samples were pre-incubated for 1 min at 37 °C. Reactions were terminated after 10 min of incubation at 37 °C by adding 25 μ l (0.25 times the incubation volume) of ice-cold ACN. The formation of the GSH conjugate of 1'-oxomyristicin (GS-1'-oxomyristicin) was linear with time and microsomal protein concentration under the experimental conditions used. Blank incubations were performed without the cofactor NAD⁺. Incubations were performed in triplicate. All

samples were centrifuged for 5 min at 13000×g, and the supernatant was stored at −20 °C until UPLC analysis.

Sulfonation of 1'-hydroxymyristicin to 1'-sulfoxymyristicin

The formation of 1'-sulfoxymyristicin was determined using incubations containing male rat liver Sprague–Dawley or pooled gender human liver S9 proteins, PAPS as a cofactor, and GSH, which acts as a scavenger of the reactive carbocation formed due to the unstable nature of the 1'-sulfoxy metabolite in an aqueous environment (van den Berg et al. 2012; Martati et al. 2011; Al-Subeihi et al. 2011). Incubation mixtures containing (final concentrations) 10 mM of GSH, 0.2 mM of PAPS, and 3 mg/ml of S9 proteins in 0.1 M potassium phosphate (pH 8.2) were pre-incubated for 1 min at 37 °C. After the pre-incubation, 1'-hydroxymyristicin dissolved in DMSO was added in final concentrations ranging between 10 and 6000 μM while keeping the final content of DMSO at 1 % (v/v). The reaction was terminated after 360 min of incubation by adding 25 μl (0.25 times the incubation volume) of ice-cold ACN. The formation of the GSH conjugate of 1'-sulfoxymyristicin was linear with time and S9 protein concentrations under the experimental conditions used. Blank incubations were performed in the absence of PAPS. Incubations were performed in triplicate. All samples were centrifuged for 5 min at 16000×g, and the supernatant was stored at −20 °C until ultra-performance liquid chromatography (UPLC) analysis.

Identification and quantification of metabolism of myristicin and 1'-hydroxymyristicin by UPLC

Microsomal incubations with myristicin only detected primary metabolites, and all incubation conditions were chosen such that substrate conversion did not exceed 10 % of the initial substrate concentration, a condition that is also essential to ascertain adequate determination of the kinetic constants. Secondary metabolism of the relevant 1'-hydroxy metabolite of myristicin was taken into account by determining the kinetic constants for its conversion in (a) glucuronidation, (b) sulfation, and (c) oxidation, again under conditions that allowed adequate definition of kinetic constants and with <10 % substrate conversion. Incubation samples were subjected to UPLC analysis that was performed as described previously (Punt et al. 2008). Identification was achieved by comparing the UV spectra of the formed metabolites to the spectra of the synthesized 1'-hydroxymyristicin and 1'-oxomyristicin reference standards. Quantification of all formed metabolites was done by comparing the peak areas to those of calibration curves of the corresponding reference compounds, determined using UPLC with photodiode array detection (UPLC-PDA). The UPLC system was composed of a Waters (Waters, Milford, MA) Acquity solvent manager, sample manager, and photodiode array detector, equipped with Water Acquity UPLC BEH C18 column.

For 2',3'-dihydroxymyristicin and 5-allyl-2,3-dihydroxyanisole that were found to have the same UV spectrum as 1'-hydroxymyristicin, and for 1'-hydroxymyristicin, estimation was based on the comparison of the peak area of the formed metabolites to the calibration curve of the synthesized 1'-hydroxymyristicin at a wavelength of 210 nm. For 3'-hydroxymyristicin, estimation was based on the comparison of the peak area to the calibration curve of the GSH adduct of synthesised 1'-oxomyristicin at a wavelength of 212 nm, because 3'-hydroxymyristicin

was found to have the same UV spectrum as the GSH adduct of synthesised 1'-oxomyristicin. The gradient for analysis of microsomal metabolites was performed with 100 % ACN and ultrapure water containing 0.1 % (v/v) TFA. The flow rate was 0.6 ml/min. The gradient started at 10 % ACN, increased to 50 % ACN over 4 min, increased to 80 % over 0.5 min, followed by decrease to 10 % ACN over 0.5 min, and finally kept at 10 % for 1 min.

1'-Hydroxymyristicin glucuronide was estimated based on the comparison of the peak area of the formed metabolite to the calibration curve of 1'-hydroxymyristicin at a wavelength of 210 nm. The flow rate was 0.6 ml/min. The gradient was made using a mixture of ACN and ultrapure water containing 0.1 % (v/v) TFA. The gradient started at 10 % ACN, increased to 60 % over 3.5 min, after which ACN was increased to 80 % over 0.5 min, and kept at 80 % for 0.5 min, and finally decreased to 10 % over 0.5 min.

Quantification of GS-1'-oxomyristicin was based on a calibration curve of the GSH adduct of the synthesized 1'-oxomyristicin made as previously described (van den Berg et al. 2012; Punt et al. 2009; Martati et al. 2011; Al-Subeihi et al. 2011). In short, a 60 μ M concentration of the synthetic standard of 1'-oxomyristicin, dissolved in ACN, was incubated with different concentrations of GSH (i.e., 0–20 μ M) in 0.2 M Tris-HCl (pH 7.4) for 6-hr at 37 °C, resulting in full conversion of the GSH to GS-1'-oxomyristicin. Quantification was done by comparing the peak areas of the formed GS-1'-oxo metabolite in the incubation mixtures with peak areas of the GS-1'-oxomyristicin calibration curve thus obtained at a wavelength of 212 nm. The gradient for analysis of the metabolite consisted of a mixture of ACN and ultrapure water containing 0.1 % (v/v) TFA. The flow rate was 0.6 ml/min, The gradient started with 10 % ACN, increased to 30 % ACN over 2.5 min, after which ACN was increased to 80 % over 0.5 min, kept at 80 % for 0.5 min, followed by a decrease to 10 % over 0.5 min, and finally kept at 10 % for 0.5 min.

Quantification of 1'-sulfoxymyristicin was done using UPLC analysis as described for the estimation of 1'-sulfoxysafrole (Martati et al. 2011). The UV spectrum of the GSH adduct of 1'-sulfoxymyristicin was found to be similar to the UV spectrum of the GSH conjugate of 1'-oxomyristicin, and estimation of the GSH adduct of 1'-sulfoxymyristicin was thus accomplished by comparing the peak area of this metabolite to the calibration curve of GS-1'-oxomyristicin at a wavelength 305 nm. The gradient for analysis of the metabolite consisted of a mixture of ACN and ultrapure water containing 0.1 % (v/v) TFA. The flow rate was 0.6 ml/min, starting at 0 % ACN and increasing the percentage of ACN to 20 % over 0.2 min, followed by an increase to 30 % ACN over 4.3 min, then increasing to 100 % over 0.3 min, and keeping it at 100 % for 0.2 min, and finally decreasing to 0 % over 0.2 min and keeping it at 0 % for 0.8 min. Separation and purification of the GSH adduct of 1'-sulfoxymyristicin was performed by collecting the peak of the metabolite from the UPLC column. Then, LC-MS analysis of the metabolite was conducted using a micro-TOF MS (Bruker) coupled to an Agilent LC (1200 Series) equipped with Altima C18 column (150 × 4.6 mm, 3 μm). The mobile phase used consisted of (A) nanopure water with 0.1 % formic acid and (B) HPLC-grade ACN with 0.1 % formic acid. Elution was at a flow rate of 0.8 ml/min, starting at 22 % B with a linear increase to 100 % B in 30 min. Subsequently, the gradient returned linearly to the initial conditions in 2 min and remained 13 min at this condition prior to the next injection. Mass spectrometric analysis was in the negative electrospray mode using a spray capillary voltage of 4500 V, a capillary temperature of 200 °C, and nitrogen as nebulizer gas at 8.0 L/min.

Determination of kinetic constants

Kinetic constants for the metabolic conversions of myristicin and 1'-hydroxymyristicin were derived by fitting the data to the standard Michaelis–Menten equation,

$$v = V_{\max} \times [S] / K_m + [S]$$

For conversion of 1'-hydroxymyristicin to 1'-sulfoxymyristicin, a first-order rate linear equation was used:

$$v = k_{HMS} \times [S]$$

In which [S] represents the substrate concentration, V_{\max} the maximum velocity, and K_m the Michaelis–Menten constant for the formation of the different metabolites of myristicin or 1'-hydroxymyristicin, k_{HMS} the first-order rate constant for sulfonation of 1'-hydroxymyristicin. Data analysis was accomplished using GraphPad Prism, version 5.04 (GraphPad Software, San Diego, CA).

Physiologically based kinetic (PBK) models

Two PBK models were developed describing the relative importance of bioactivation and detoxification of myristicin in rat and human at different oral dose levels. The models developed in this study were essentially based on the PBK models previously defined for the metabolism of estragole (Punt et al. 2008, 2009), methyleugenol (Al-Subeihi et al. 2011, 2012), elemicin (van den Berg et al. 2012), and safrole (Martati et al. 2011, 2012) in rat and human. A schematic overview of the developed PBK models for myristicin metabolism in rat and human is shown in Figure 3. The models consist of several compartments representing different organs and tissues (i.e., liver, fat tissue, richly perfused tissues, and slowly perfused tissues) that are mutually connected through the systemic circulation. First-order kinetics was used to describe the uptake

of myristicin from the gastrointestinal (GI) tract assuming a direct uptake by the liver with an absorption rate constant (k_a) of 1.0 h^{-1} , which is based on the fast and complete absorption of the structurally related alkenylbenzene safrole from the GI tract (Punt et al. 2008). For rat and human, the liver was the only organ able to convert myristicin to different microsomal metabolites. 2',3'-Dihydroxymyristicin (DHM), 3'-hydroxymyristicin (3HM), 1'-hydroxymyristicin (HM), and 5-allyl-2,3-dihydroxyanisole (DHA) were formed in incubations with both rat and human liver.

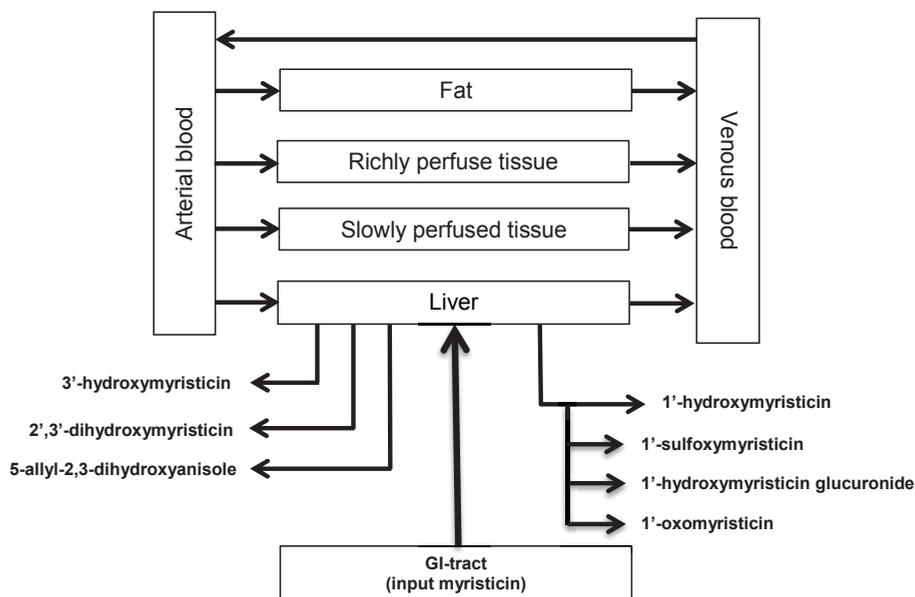


Figure 3. Schematic overview of the PBK model for myristicin in rat and human.

Accordingly, mass balance equations for myristicin in rat liver and human were as follows:

$$\begin{aligned} \partial A_{LM}/\partial t &= \partial Uptake_M/\partial t + QL \times (CA_M - CL_M/PL_M) \\ &\quad - V_{max_{HM}} \times CL_M/PL_M / (K_{m_{HM}} + CL_M/PL_M) \\ &\quad - V_{max_{3HM}} \times CL_M/PL_M / (K_{m_{3HM}} + CL_M/PL_M) \\ &\quad - V_{max_{DHA}} \times CL_M/PL_M / (K_{m_{DHA}} + CL_M/PL_M) \\ &\quad - V_{max_{DHM}} \times CL_M/PL_M / (K_{m_{DHM}} + CL_M/PL_M) \\ \partial Uptake/\partial t &= -\partial AGI_M/\partial t = K_a \times AGI_M, AGI_M(0) = oral\ dose \\ CL_M &= A_{LM}/VL \end{aligned}$$

Where $Uptake_M$ (μmol) is the amount of myristicin taken up from the GI tract, AGI_M (μmol) is the amount of myristicin remaining in the GI tract, and A_{LM} (μmol) is the amount of myristicin in the liver or CL_M is the myristicin concentration in the liver ($\mu\text{mol/L}$). CA_M and CV_M are the myristicin concentrations in the arterial and venous blood (both in $\mu\text{mol/L}$), QL is the blood flow rate to a tissue (L/h), QC is the cardiac output (L/h), VL is the volume of liver, PL_M is the tissue/blood partition coefficient of myristicin, and V_{max} and K_m are the values representing the maximum rate of formation and Michaelis–Menten constant, respectively, for the formation of 2',3'-dihydroxymyristicin (DHM), 3'-hydroxymyristicin (3HM), 1'-hydroxymyristicin (HM), and 5-allyl-2,3-dihydroxyanisole (DHA).

The mass balance equation for the metabolic conversion of 1'-hydroxymyristicin by glucuronidation, oxidation, and sulfonation in the liver in rat and human liver is as follows:

$$\begin{aligned} \partial A_{LM}/\partial t &= V_{max_{HM}} \times CL_M/PL_M / (K_{m_{HM}} + CL_M/PL_M) \\ &\quad - V_{max_{L_{HMG}}} \times CL_{HM}/PL_{HM} / (K_{m_{HMG}} + CL_{HM}/PL_{HM}) \\ &\quad - V_{max_{HMO}} \times CL_{HM}/PL_{HM} / (K_{m_{HMO}} + CL_{HM}/PL_{HM}) \\ &\quad - k_{HMS} \times CL_{HM}/PL_{HM} \\ CL_M &= A_{LM}/VL \end{aligned}$$

where AL_{HM} is the amount of 1'-hydroxymyristicin in the liver (μmol), CL_{HM} is the 1'-hydroxymyristicin concentration in the liver ($\mu\text{mol/L}$), PL_{HM} is the liver/blood partition coefficient of 1'-hydroxymyristicin, and V_{max} and K_m are the maximum rate of formation and Michaelis–Menten constant, respectively, for the formation of the different 1'-hydroxymyristicin metabolites in the liver, including 1'-hydroxymyristicin glucuronide, 1'-oxomyristicin, and k_{HMS} is the first-order rate constant for sulfonation of 1'-hydroxymyristicin that was used instead of K_m and V_{max} as sulfonation showed no saturation. V_{max} and K_m values and first-order rate constants k in case of the absence of saturation (for sulfonation of 1'-hydroxymyristicin in rat and human) for the different metabolic pathways of myristicin and 1'-hydroxymyristicin were derived from results from *in vitro* experiments in the present study. V_{max} values that were derived *in vitro* expressed as $\text{nmol min}^{-1} (\text{mg liver microsomal or S9 protein})^{-1}$ were scaled to values representing the V_{max} per $\mu\text{mol h}^{-1} (\text{g liver})^{-1}$ using microsomal protein yields of 35 mg/g for rat and 32 mg/g for human liver and S9 protein yields of 143 mg/g for liver, as defined previously (Punt et al. 2008, 2009), based on Medinsky et al. (1994). First-order rate constant k expressed in $\text{ml min}^{-1} (\text{mg liver S9 protein})^{-1}$ was scaled to values expressed in ml h^{-1} per g liver using the same conversion factor for S9 protein yield. Tables 2 and 3 summarize the physiological parameters (i.e., tissue volumes, cardiac output, and tissue blood flows) for rat and human, respectively, which were derived from the literature (Brown et al. 1997). Partition coefficients were derived *in silico* based on a method described by DeJongh et al. (1997) using the log K_{ow} . Log K_{ow} values for myristicin (Clog P 3.1721) and 1'-hydroxymyristicin (Clog P 1.6151) were estimated using Chemdraw professional 15 (ChemOffice® Professional 15.0 by perkin elmer). Mass balance equations were coded and numerically integrated in Berkely Madonna 8.3.18 (Macey and Oster, UC Berkeley, CA) using Rosenbrock's algorithm for stiff systems. PBK

models in rat and human liver were run for 720-hr, because that would be the time for total clearance of myristicin in human tissues after one dose.

Table 2. Parameters used in the physiologically based kinetic model for myristicin in male rat.

Physiological parameters (Brown, 1997)		Tissue: blood partition coefficients	
body weight (kg)	0.25	myristicin	
percentage of body weight		liver	
liver	3.4	fat	1.68
fat	7.0	rapidly perfused	37.73
rapidly perfused	5.1	slowly perfused	1.68
slowly perfused	60.2		0.67
blood	7.4	1'-hydroxymyristicin	
		liver	0.94
cardiac output (L/h)	5.4		
percentage of cardiac output			
liver	25		
fat	7.0		
rapidly perfused	51.0		
slowly perfused	17.0		

Table 3. Parameters used in the physiologically based kinetic model for myristicin in human.

Physiological parameters (Brown, 1997)		Tissue: blood partition coefficients	
body weight (Kg)	60	myristicin	
percentage of body weight		liver	4.22
liver	2.6	fat	83.45
fat	21.4	rapidly perfused	4.22
rapidly perfused	5.0	slowly perfused	2.81
slowly perfused	51.7		
blood	7.9	1'-hydroxymyristicin	
		liver	0.93
cardiac output (L/h)	310		
percentage of cardiac output			
liver	22.7		
fat	5.2		
rapidly perfused	47.3		

Sensitivity analysis

To determine which parameters have the greatest influence on model predictions, a sensitivity analysis was performed as described previously (Punt et al. 2008, 2009; van den Berg et al. 2012; Martati et al. 2011, 2012; Al-Subeihi et al. 2011, 2012). For this purpose, normalized sensitivity coefficients (SCs) were determined using the following equation:

$$SC = (C' - C)/(P' - P) \times (P/C)$$

where C is the initial value of the model output, C' is the modified value of the model output resulting from an increase in parameter value, P is the initial parameter value, and P' represents the modified parameter value. An increase of 5 % in parameter values was used to analyze the effect of a change in parameter on the formation of 1'-hydroxymyristicin and 1'-

sulfoxymyristicin (expressed as a percentage of the dose). Each parameter was analyzed individually, while the other parameters were kept at their initial value.

Comparison of the PBK model-based prediction of bioactivation of myristicin to the PBK model-based predictions for bioactivation of the structurally related compound safrole

The predicted model outcomes for the formation of 1'-hydroxymyristicin and 1'-sulfoxymyristicin in the liver of rat and human were compared with the predicted dose-dependent formation of the 1'-hydroxy- and 1'-sulfoxy metabolite of the structurally related alkenylbenzene safrole. For this purpose, the previously defined PBK models for safrole described by Martati et al. (2011, 2012) for rat and human were used (Martati et al. 2011, 2012). For the comparison, the models describing the metabolism of myristicin and safrole were run for a period of 720-hr.

RESULTS

Microsomal conversion of myristicin

To identify which organs are involved in the metabolism of myristicin in both male rat and human, incubations were performed using microsomal protein preparations from liver, kidney, lung, and small intestine of both species. Chromatographic analysis of these incubations revealed that for rat and human, no detectable metabolism of myristicin occurred in small intestine, lung, or kidney microsomes, and metabolism was only observed for liver microsomes.

An example of a chromatogram of an incubation of myristicin with male rat liver microsomes and NADPH as a cofactor is shown in Figure 4. In incubations with rat liver microsomes, 2',3'-dihydroxymyristicin ($R_t = 1.19$ min), 3'-hydroxymyristicin ($R_t = 2.01$ min), 1'-hydroxymyristicin

($R_t = 2.04$ min), and 5-allyl-2,3-dihydroxyanisole ($R_t = 2.11$ min) were formed. In incubations with human liver microsomes, the same four metabolites were found. Identification was done based on the comparison of the UV spectra and retention times of the formed metabolites with those of the specific synthesized or commercially available reference compounds. However, tentative identification of 2',3'-dihydroxymyristicin was based on the fact that the epoxidation of the double bond in the allyl side chain of estragole, methyleugenol, and safrole yields the 2',3'-epoxide, found to be hydrolyzed by epoxide hydrolase to the 2',3'-dihydroxy metabolite (Luo et al. 1992; Luo and Guenther 1996; Guenther and Luo 2001). The hydrolysis product of the epoxide, 2',3'-dihydroxy metabolite of myristicin, safrole, or elemicin, was detected in the urine of rats administered high doses (100 mg/kg bw) of each substance individually or a high dose of nutmeg (500 mg/kg bw) (Beyer et al. 2006). Data revealed that 3'-hydroxymyristicin was formed directly from myristicin rather than from isomerization of 1'-hydroxymyristicin since the formation of 3'-hydroxymyristicin was not observed in incubations of 1'-hydroxymyristicin with liver microsomes and NADPH (data not shown). The kinetic constants (i.e., V_{max} and K_m) were derived from plots of the rates of formation of different microsomal metabolites in incubations with male rat liver microsomes, and human liver microsomes from myristicin at concentrations ranging from 25 to 1000 μM (for details, see Figure S1 in the supplementary materials). The values obtained are shown in Table 4, together with the catalytic efficiencies, calculated as V_{max}/K_m . Analysis of incubations that were performed with male rat liver microsomal preparations revealed that the metabolite arising from *O*-demethylenation of myristicin, namely 5-allyl-2,3-dihydroxyanisole, was formed with the highest V_{max} value. Moreover, the 2',3'-dihydroxymyristicin and 5-allyl-2,3-dihydroxyanisole were abundantly formed in incubations with male rat microsomal liver preparations with a high affinity. In incubations performed with

male rat liver microsomes, 1'-hydroxymyristicin and 3'-hydroxymyristicin were the least important metabolites formed upon conversion of myristicin. In general, the catalytic efficiency for the formation of 5-allyl-2,3-dihydroxyanisole by male rat liver microsomes had the highest value, followed by the catalytic efficiency for the formation of 2',3'-dihydroxymyristicin, 1'-hydroxymyristicin, and 3'-hydroxymyristicin, respectively. The catalytic efficiency for the formation of 5-allyl-2,3-dihydroxyanisole was found to be approximately 7-fold higher than that for the formation of 3'-hydroxymyristicin. In incubations with human liver fractions, 5-allyl-2,3-dihydroxyanisole was found to be the most abundant metabolite formed followed by 2',3'-dihydroxymyristicin, 1'-hydroxymyristicin, and 3'-hydroxymyristicin, respectively. Analysis of the catalytic efficiencies for the formation of the different microsomal metabolites of myristicin, obtained with pooled human liver microsomes, showed that the formations of 2',3'-dihydroxymyristicin, 1'-hydroxymyristicin, and 3'-hydroxymyristicin were the least important routes of myristicin metabolism, whereas the formation of 5-allyl-2,3-dihydroxyanisole represents the major pathway for the human liver microsomal conversion of myristicin.

Glucuronidation of 1'-hydroxymyristicin

Chromatographic analysis of incubations with male rat and mixed gender pooled human liver S9, UDPGA as cofactor and 1'-hydroxymyristicin as substrate, revealed a peak at 1.36 min (chromatogram not shown). Moreover, chromatographic analysis of incubations performed in the absence of the cofactor UDPGA did not show a peak at a retention time of 1.36 min. Together, these data indicate that the compound eluting at 1.36 min can be assumed to be 1'-hydroxymyristicin glucuronide. The rate of the metabolic conversion of 1'-hydroxymyristicin to 1'-hydroxymyristicin glucuronide in incubations with both male rat and human liver fractions

with increasing concentrations of 1'-hydroxymyristicin is presented in Figure 5a, d, respectively.

The kinetic constants derived from these plots are presented in Table 4.

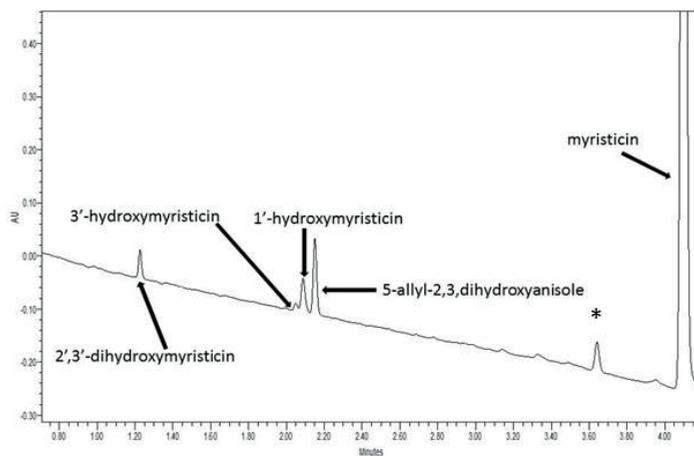


Figure 4. UPLC chromatogram of an incubation of myristicin (1000 μ M) with male rat liver microsomes. The peak marked with an asterisk (*) was also found in the blank incubation without NADPH.

Table 4. Kinetic constants for metabolism of myristicin and 1'-hydroxymyristicin as derived from data obtained in incubations with Sprague-Dawley male rat liver microsomes and mixed gender pooled human liver microsomes and respective cofactors.

SPECIES		RAT			HUMAN				
Metabolite	Abbreviation	$K_m^{a,c}$	$V_{max}^{a,b}$	<i>in vitro</i> catalytic efficiency ^d	$k_{\pm SD}$	$K_m^{a,c}$	$V_{max}^{a,b}$	<i>in vitro</i> catalytic efficiency ^d	$k_{\pm SD}$
conversion of myristicin									
2',3'-dihydroxymyristicin	DHM	16±7	0.44±0.08	27.5		33±12	0.08±0.01	2.424	
3'-hydroxymyristicin	3HM	83±31	0.47±0.09	5.663		1629±1545	0.29±0.14	0.178	
1'-hydroxymyristicin	HM	102±32	0.86±0.18	8.431		234±132	0.17±0.03	0.726	
5-allyl-2,3-dihydroxyanisole	DHA	33±11	1.3±0.23	39.394		118±36	0.93±0.13	7.881	
conversion of 1'-hydroxymyristicin									
1'-hydroxymyristicin glucuronide ^e	HMG	836±336	8.6±2.0	10.287		3140±2434	0.08±0.03	0.025	
1'-oxomyristicin	HMO	5922±3477	43.64±20.9	7.369		3244±2646	32±14	9.864	
1'-sulfoxymyristicin ^e	HSM				17.6×10 ⁻⁶				16.3×10 ⁻⁶

^a Mean values of three independent measurements±SD. ^b nmol min⁻¹ (mg microsomal or S9 protein)⁻¹. ^c μM. ^d μL min⁻¹ (mg microsomal or S9 protein)⁻¹ (V_{max}/K_m × 1000 μL/mL). ^e Experiments performed with S9 tissue fractions.^f first order rate constant for product formation(ml/min/mg protien)

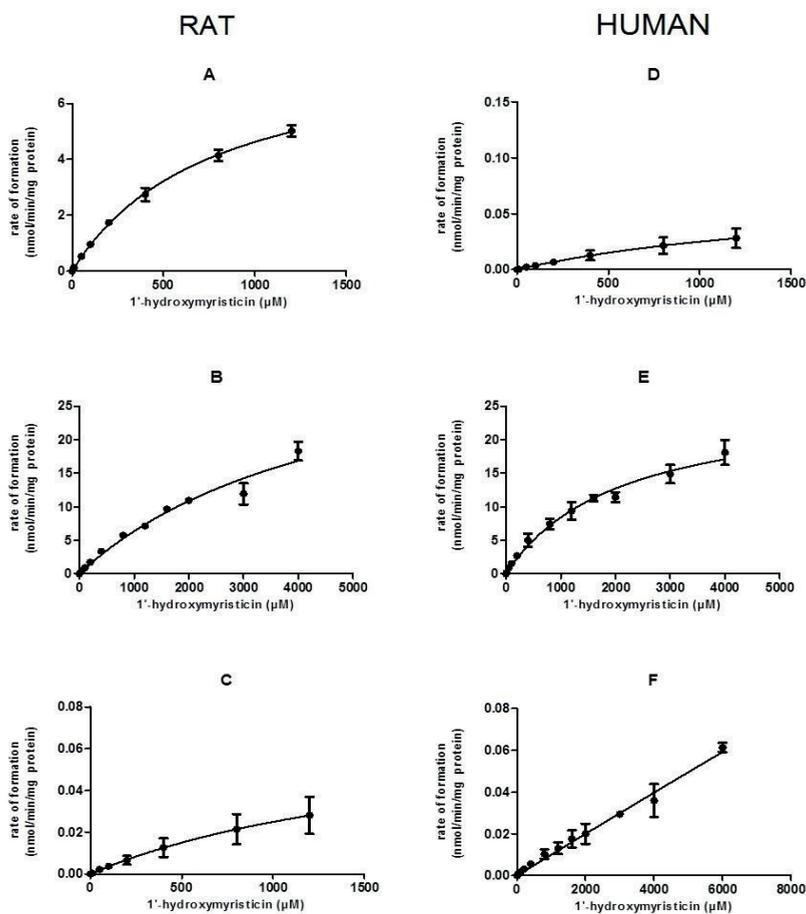


Figure 5. Concentration-dependent rate of (A) glucuronidation of 1'-hydroxymyristicin in incubations with male rat liver S9 or (D) pooled human mixed gender liver S9, (B) oxidation of 1'-hydroxymyristicin in incubations with pooled male rat liver microsomes or (E) pooled human mixed gender liver S9, and (C) sulfonation of 1'-hydroxymyristicin in pooled male rat liver S9 or (F) pooled human mixed gender liver S9. Data points represent mean values \pm SD of three or four individual experiments.

Oxidation of 1'-hydroxymyristicin

The rate of oxidation of 1'-hydroxymyristicin with increasing concentrations of 1'-hydroxymyristicin in incubations with male rat and pooled human liver microsomes is shown in Figure 5b, e, respectively, and the kinetic constants derived from these data are presented in Table 4.

Sulfonation of 1'-hydroxymyristicin

In the present study, GSH was used to trap the reactive 1'-sulfoxymyristicin formed upon sulfonation of the proximate carcinogenic metabolite of myristicin, 1'-hydroxymyristicin. The scavenging is based on a chemical reaction but can also be catalyzed by the GST present in the S9 incubations in which the sulfonation of 1'-hydroxymyristicin was measured. Chromatographic analysis of incubations with male rat or mixed gender pooled human liver S9, increasing concentration of 1'-hydroxymyristicin, PAPS, and GSH revealed a peak at 1.05 min, which was identified as the GSH adduct of the carbocation of 1'-sulfoxymyristicin. Identification was achieved based on the chromatographic analysis of incubations performed in the absence of GSH and the presence of PAPS and liver S9 proteins since in these incubations; no peak was found at 1.05 min. The identity was verified by LC-MS analysis, which revealed a deprotonated molecule at m/z 496 that corresponds to the expected $[M-H]$ mass of a GSH adduct of 1'-sulfoxymyristicin. The rate of formation of 1'-sulfoxymyristicin in incubations with male rat and mixed gender pooled human liver S9 is presented in Figure 5c, f, respectively. The kinetic constants for the conversion of 1'-hydroxymyristicin to 1'-sulfoxymyristicin in rat and human liver are presented in Table 4.

Comparison of the kinetic constants for conversion of myristicin and 1'-hydroxymyristicin by male rat and mixed gender pooled human tissue fractions

To allow a comparison between the kinetic constants for metabolism of myristicin and 1'-hydroxymyristicin by male rat and mixed gender pooled human tissue fractions, V_{\max} values that were derived *in vitro* expressed in $\text{nmol min}^{-1} (\text{mg microsomal or S9 protein})^{-1}$ were scaled to values representing the V_{\max} per $\mu\text{mol h}^{-1} (\text{g tissue})^{-1}$ using microsomal and S9 protein yields as described in the literature (Medinsky et al. 1994) and previously used (Martati et al. 2011, 2012; van den Berg et al. 2012; Punt et al. 2009; Al-Subeihi et al. 2011, 2012). Making the use of the *in vivo* V_{\max} values derived accordingly, a scaled catalytic efficiency (scaled V_{\max}/K_m) for the formation of myristicin metabolites could be calculated (Table 5). For sulfonation, the rate constants for product formation (ml/min/mg protein) were scaled based on S9 protein yield of 143 mg/g liver. These values show that the catalytic efficiency for the formation of the proximate carcinogenic metabolite of myristicin, 1'-hydroxymyristicin, was found to be 13-fold higher in male rat liver as compared to human liver. This difference in catalytic efficiency for the formation of 1'-hydroxymyristicin is predominantly caused by the high affinity (expressed as K_m) and high activity (expressed as V_{\max}) for its formation from myristicin in male rat liver, since the K_m value in rat liver incubations was 2-fold lower than that in human liver incubations and V_{\max} in rat liver incubations was 5-fold higher than that in human liver incubations. The detoxification of 1'-hydroxymyristicin by formation of 1'-hydroxymyristicin glucuronide was found to be the main metabolic reaction with 1'-hydroxymyristicin in rat. Glucuronidation of 1'-hydroxymyristicin occurs in male rat with higher affinity than in human (i.e., K_m 836 μM), whereas in human the K_m was 3140 μM , and analysis of the data revealed a high V_{\max} value for 1'-hydroxymyristicin glucuronidation resulting in a catalytic efficiency that was 400-fold higher

in male rats as compared to human. Oxidation of 1'-hydroxymyristicin was found to be 1.2 times more efficient in human liver as compared to male rat liver resulting from a 1.8-fold lower affinity in rat. Sulfonation was found to be the least efficient metabolic pathway for 1'-hydroxymyristicin in both rats and human. For rat, the *in vivo* scaled k was $0.15 \text{ ml h}^{-1} (\text{g tissue})^{-1}$, and for human, it was $0.14 \text{ ml h}^{-1} (\text{g tissue})^{-1}$, indicating that sulfonation of 1'-hydroxymyristicin is equally efficient in male rat liver and human liver. Altogether, it can be concluded that glucuronidation of 1'-hydroxymyristicin, representing a detoxification pathway, is the most important pathway in rat, and the oxidation is the most important pathway for conversion of 1'-hydroxymyristicin in human. Moreover, on the basis of the kinetic data obtained, bioactivation of 1'-hydroxymyristicin following sulfonation was found to represent only a minor pathway in both rat and human.

Evaluation of PBK model performance

The performance of the newly developed PBK models for myristicin could not be evaluated against *in vivo* data because quantitative data on the formation or excretion of the different metabolites in rat or humans exposed to myristicin are not available. However, Beyer et al. (2006) reported that in rats and humans, myristicin and safrole were *O*-demethylenated. In the urine of the human nutmeg abuser, who had taken 5 nutmegs, the acetylated metabolites of 5-allyl-2,3-dihydroxyanisole and 2',3'-dihydroxymyristicin could be identified. In the same study, *O*-demethylenation of myristicin to generate 5-allyl-2,3-dihydroxyanisole seems to result in the main metabolite of myristicin detected in rat urine samples collected over a 24-hr period after administration of a single oral dose of 100 mg/kg bw myristicin, with a percentage of 67 % of

Table 5. Scaled kinetic constants for metabolic conversion of myristicin and 1'-hydroxymyristicin by male rat and human tissue fractions.

SPECIES		RAT ^a				HUMAN ^a				
Metabolite	Abbreviation	scaled Vmax, <i>in vivo</i> (μmol/h/g tissue) ^b	K _m (μM)	<i>in vivo</i> catalytic efficiency (mL/h/g tissue) ^c	k scaled (mL/hr/g tissue) ^d	scaled Vmax, <i>in vivo</i> (μmol/h/g tissue) ^b	K _m (μM)	<i>in vivo</i> catalytic efficiency (mL/h/g tissue) ^c	k scaled (mL/hr/g tissue) ^d	
conversion of myristicin										
2',3'-dihydroxymyristicin	DHM	0.924	16±7	0.0578		0.154	33±12	0.0047		
3'-hydroxymyristicin	3HM	0.987	83±31	0.0119		0.557	1629±1545	0.0003		
1'-hydroxymyristicin	HM	1.806	102±32	0.0177		0.326	234±132	0.0014		
5-allyl-2,3-dihydroxyanisole	DHA	2.73	33±11	0.0827		1.786	118±36	0.0151		
conversion of 1'-hydroxymyristicin										
1'-hydroxymyristicin glucuronide	HMG	73.788	836±336	0.0883		0.6864	3140±2434	0.0002		
1'-oxomyristicin	HMO	91.644	5922±3477	0.0155		61.44	3244±2646	0.0189		
1'-sulfoxymyristicin	HMS				0.15				0.14	

^a Mean values of three independent measurements. ^b Scaled Vmax values were converted from *in vitro* Vmax values based on microsomal protein yields of 35 and 32 mg/g tissue liver for rat and human, respectively, and a S9 protein yield of 143 mg/g liver. ^c Catalytic efficiency (scaled Vmax (app)/ Km). ^d k scaled based on S9 protein yield of 143 mg/g liver.

the total dose (Beyer et al. 2006). In line with these results, the developed PBK model predicted 5-allyl-2,3-dihydroxyanisole to be the major metabolite formed at a dose of 100 mg/kg bw myristicin in rat after 24-hr, with a percentage of 73 % of the total dose. This predicted value of 73 % of the dose matches well with the 67 % observed in the *in vivo* rat study.

Important to note is that the PBK models for myristicin were based on the proposed biotransformation in Figure 2 and comparable with the PBK models for estragole, methyleugenol, and safrole, for which more data allowing the evaluation of the models were available. The performance of the rat PBK models developed for estragole, methyleugenol, and safrole was reported before (Punt et al. 2008; Martati et al. 2011; Al-Subeihi et al. 2011). Evaluation was done by comparing the predicted levels of a variety of metabolites in plasma or excreted in the urine of rats. Data revealed that the predicted PBK model values and the levels of these metabolites derived from *in vivo* studies adequately matched (Punt et al. 2008; Martati et al. 2011; Al-Subeihi et al. 2011). Furthermore, also for the developed human PBK models for estragole (Punt et al. 2009), methyleugenol (Al-Subeihi et al. 2012), and safrole (Martati et al. 2012), a comparison could be made between the model predictions and the reported *in vivo* data for blood concentrations or the urinary excretion of some of the metabolites, thereby further supporting the validity of the models. Considering these data, it was concluded that the developed PBK models for myristicin will also adequately describe the *in vivo* levels of metabolites formed in rat and human after conversion of myristicin and 1'-hydroxymyristicin at different oral dose levels of myristicin.

PBK model predictions

PBK modeling was performed at dose levels of 0.05 and 300 mg/kg bw to allow comparison with the PBK model outcomes previously reported for safrole (Martati et al. 2011, 2012).

Following an exposure to 0.05 mg/kg bw myristicin, both myristicin and its proximate carcinogenic metabolite 1'-hydroxymyristicin were predicted and observed to be almost completely metabolized within a 720-hr period in rat and human. At a higher oral dose level of 300 mg/kg bw myristicin, both myristicin and 1'-hydroxymyristicin were also predicted to be fully metabolized within 720-hr. Therefore, for all further modelings, values were calculated at 720-hr after dosing. Figure 6a shows the PBK model-based predictions for the dose-dependent formation of the different microsomal metabolites of myristicin in rat. The percentage of the dose converted to 1'-hydroxymyristicin is predicted to increase in a dose-dependent manner. Concurrent with the increased percentage of the dose that undergoes 1'-hydroxylation of the alkene side chain, a 1.6-fold dose-dependent decrease in the percentage of the dose that underwent epoxidation to give 2',3'-dihydroxymyristicin was observed comparing the levels at 0.05 and 300 mg/kg bw. At the same time, a <1.6-fold increase in the formation of 3'-hydroxymyristicin was observed. The formation of 5-allyl-2,3-dihydroxyanisole did not change with increasing dose levels and was equal to 48 % of the administered dose. Figure 6b shows the dose-dependent increase in the formation of the metabolites of 1'-hydroxymyristicin in rat. This reveals a 1.8-fold increase in the percentage of the dose ultimately converted into 1'-hydroxymyristicin glucuronide, 1'-oxomyristicin, and 1'-sulfoxymyristicin, going from a dose of 0.05 to 300 mg/kg bw. This dose-dependent increase in the formation of the different 1'-hydroxymyristicin metabolites can be explained by the 1.8-fold increase in the formation of 1'-hydroxymyristicin with increasing dose levels. Figure 7a reveals that in human, the percentage of the dose converted to 1'-hydroxymyristicin equal 6.5 % at a dose of 0.05 mg/kg bw and increased to 8.0 % at a dose of 300 mg/kg bw. Accompanying the increase in the formation of 1'-hydroxymyristicin, there were a 1.4-fold decrease in the formation of 2',3'-dihydroxymyristicin and a steady formation of 5-allyl-2,3-dihydroxyanisole. Formation of 3'-hydroxymyristicin was predicted to increase 1.8-fold with

increasing dose from 0.05 to 300 mg/kg bw. Figure 7b shows a 1.25-fold dose-dependent increase in the percentage formation of 1'-hydroxymyristicin glucuronide, 1'-sulfoxymyristicin, and 1'-oxomyristicin, in human liver with increasing dose from 0.05 to 300 mg/kg bw. Comparison of the relative extent of bioactivation of myristicin by rat and human liver revealed that formation of 1'-hydroxymyristicin (expressed as nmol/g liver) is comparable in rat and human liver at the low dose of 0.05 mg/kg bw and 1.8-fold higher in rat liver than in human liver at a dose of 300 mg/kg bw (Figure 8a). Formation of 1'-sulfoxymyristicin (expressed as nmol/g liver) is 4-fold higher in human liver than in rat liver at a low dose of 0.05 mg/kg bw and 2.8-fold lower in rat liver than in human liver at a dose of 300 mg/kg bw (Figure 8b).

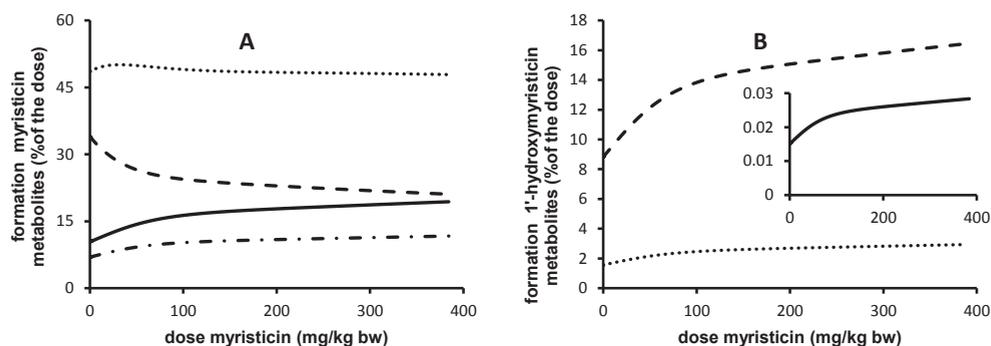


Figure 6. PBK-predicted dose-dependent changes in overall formation of (A) microsomal metabolites of myristicin in rat liver and (B) metabolites of 1'-hydroxymyristicin in rat liver. The lines correspond to (A) 5-allyl-2,3-dihydroxyanisole (••••), 3'-hydroxymyristicin (—•—), 1'-hydroxymyristicin (—), and 2',3'-dihydroxymyristicin (— —), and (B) 1'-hydroxymyristicin glucuronide (— —), 1'-sulfoxymyristicin (—), and 1'-oxomyristicin (••••)

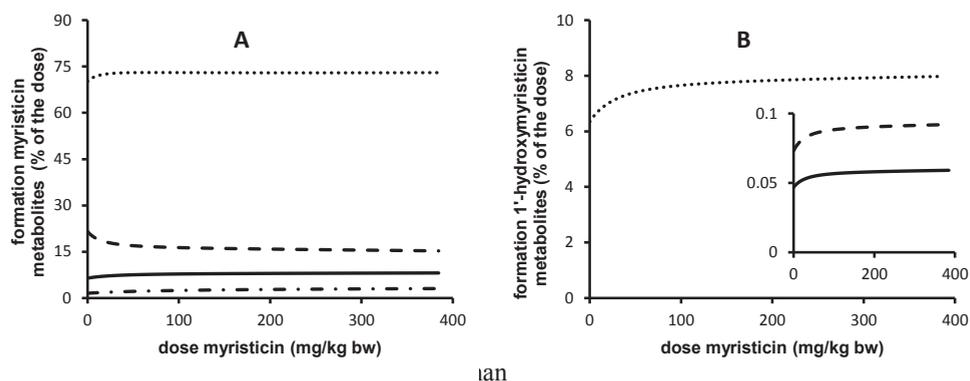


Fig. 7 PBK-predicted dose-dependent changes in overall formation of (A) microsomal metabolites of myristicin in human liver and (B) metabolites of 1'-hydroxymyristicin in human liver. The lines correspond to (A) 5-allyl-2,3-dihydroxyanisole (••••), 3'-hydroxymyristicin (— • —), 1'-hydroxymyristicin (—), and 2',3'-dihydroxymyristicin (— —), and (B) 1'-hydroxymyristicin glucuronide (— —), 1'-sulfoxymyristicin (—), and 1'-oxomyristicin (••••).

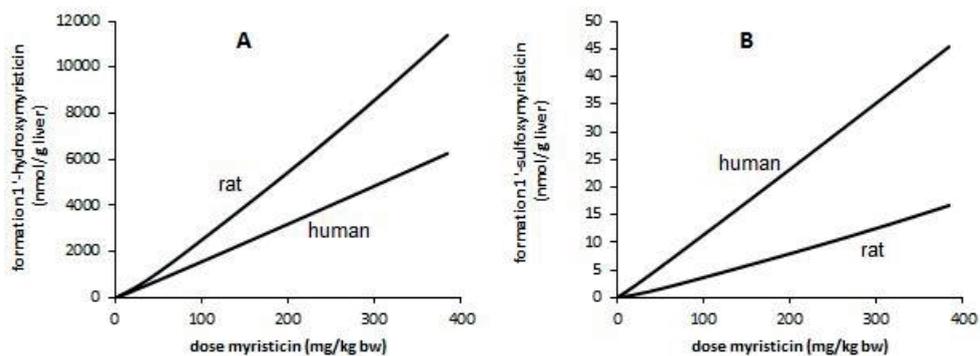


Figure 8. PBK-predicted dose-dependent formation (mg/kg bw) of (A) 1'-hydroxymyristicin and (B) 1'-sulfoxymyristicin in rat and human liver.

Sensitivity analysis

A sensitivity analysis was performed to define model parameters that are capable of influencing the formation of 1'-hydroxymyristicin and 1'-sulfoxymyristicin in rat and human liver. For this purpose, normalized sensitivity coefficients (SCs) were calculated for all parameters at a dose of 0.05 mg/kg bw myristicin. This sensitivity analysis reveals to what extent small variation in the respective parameters influences the results. The sensitivity analysis also reveals to which parameters the predicted outcomes are most sensitive. The results of this analysis are presented in Figure 9. Figure 9 especially presents the parameters affecting the formation of 1'-hydroxymyristicin (black) and 1'-sulfoxymyristicin (gray) that have a normalized SC > |0.1|. In rat liver, the formation of the ultimate carcinogenic metabolite 1'-sulfoxymyristicin is primarily influenced by the kinetic constants of 1'-hydroxymyristicin formation from myristicin ($V_{\max \text{ HM}}$, $K_{\text{m HM}}$). The kinetic constants for the formation of 5-allyl-2,3-dihydroxyanisole and 2',3'-dihydroxymyristicin ($V_{\max \text{ DHA}}$, $K_{\text{m DHA}}$, $V_{\max \text{ DHM}}$, $K_{\text{m DHM}}$) were also found to highly influence the formation of 1'-hydroxymyristicin in rat and human liver. In rat liver, the formation of the ultimate carcinogenic metabolite 1'-sulfoxymyristicin is primarily influenced by the kinetic constants of 1'-hydroxymyristicin formation from myristicin ($V_{\max \text{ HM}}$, $K_{\text{m HM}}$), and the kinetic constants for the formation of 1'-hydroxymyristicin glucuronide ($V_{\max \text{ HMG}}$, $K_{\text{m HMG}}$), k , the first-order rate constant for the sulfonation of 1'-hydroxymyristicin (k_{HMS}), and the kinetic constants for the formation of 5-allyl-2,3-dihydroxyanisole and 2',3'-dihydroxymyristicin ($V_{\max \text{ DHA}}$, $K_{\text{m DHA}}$, $V_{\max \text{ DHM}}$, $K_{\text{m DHM}}$) were also found to highly influence the formation of 1'-sulfoxymyristicin in rat liver. In human liver, the formation of the ultimate carcinogenic metabolite 1'-sulfoxymyristicin is primarily influenced by the kinetic constants of 1'-hydroxymyristicin formation from myristicin ($V_{\max \text{ HM}}$, $K_{\text{m HM}}$), the kinetic constants for the formation of 1'-oxomyristicin ($V_{\max \text{ HMO}}$, $K_{\text{m HMO}}$), k , the first-order rate constant for the sulfonation of 1'-hydroxymyristicin

(k_{HMS}) and liver microsomal protein yield (MP), and the kinetic constants for the formation of 5-allyl-2,3-dihydroxyanisole and 2',3'-dihydroxymyristicin ($V_{max\ DHA}$, $K_m\ DHA$) were also found to highly influence the formation of 1'-sulfoxymyristicin in human liver. The kinetic constants for the formation of 1'-hydroxymyristicin glucuronide were found to highly influence the formation of 1'-sulfoxymyristicin in rat liver, and the kinetic constants for formation of 1'-oxomyristicin were predicted to affect the formation of 1'-sulfoxymyristicin in human liver to a high extent. These results reflect the fact that glucuronidation of 1'-hydroxymyristicin in rat and oxidation of 1'-hydroxymyristicin in human are considered as the most important competitive metabolic pathways to sulfonation.

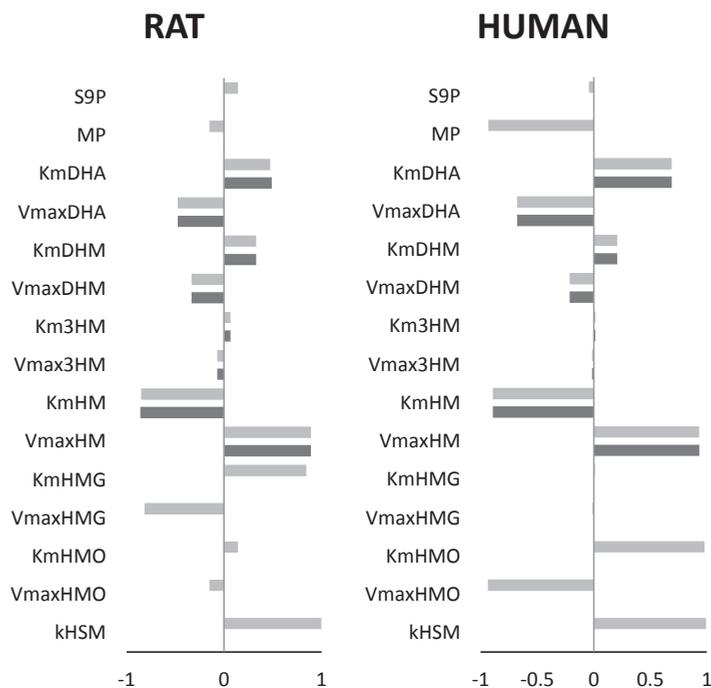


Figure 9. Normalized sensitivity coefficients for the formation from 1'-hydroxymyristicin (black) and 1'-sulfoxymyristicin (grey) in the liver of rat or human.

Comparison of the PBK model-based prediction of bioactivation of myristicin by rat and human to that of its structurally related compound safrole

In a next step, the mode of action-based PBK models for myristicin metabolism in rat and human were used to facilitate a read-across from data on safrole (Martati et al. 2011, 2012). On the basis of the PBK models, a comparison was made for the dose-dependent formation of the proximate carcinogenic 1'-hydroxy metabolite and of the ultimate carcinogenic 1'-sulfoxy metabolite of myristicin and safrole in the liver of rats. Figure 10 shows the dose-dependent formation of these metabolites in rat liver as predicted by the respective PBK models. The PBK model-based predicted formation of the proximate carcinogenic 1'-hydroxy metabolites shows that the formation of the 1'-hydroxy metabolites of myristicin and safrole was predicted to be the same at low dose 0.05 mg/kg bw and 1.4-fold higher for myristicin than that of safrole at dose level 100 mg/kg bw (Figure 10a). The predicted model outcomes for the formation of the ultimate carcinogenic 1'-sulfoxy metabolites of safrole and myristicin are shown in Figure 10b. The PBK models for rat predict the formation of 1'-sulfoxy myristicin to be 1.5-fold higher for myristicin than safrole at low dose of 0.05 mg/kg bw and 2.2-fold higher for myristicin than for safrole at dose level of 100 mg/kg bw. Figure 11 shows the predicted dose-dependent formation of 1'-hydroxy metabolites and 1'-sulfoxy metabolites of safrole and myristicin in human liver. In human liver, the predicted formation of 1'-hydroxy myristicin is 7-fold lower than the formation of 1'-hydroxy safrole at a dose level of 0.05 mg/kg bw and 4.5-fold lower for myristicin than for safrole at high dose of 100 mg/kg bw (Figure 11a). The data also reveal that in human liver, the formation of the DNA-reactive 1'-sulfoxy metabolite is comparable, 1.35-fold higher for safrole at low-dose level 0.05 mg/kg bw, and 1.1-fold higher for myristicin at high-dose level 100 mg/kg bw (Figure 11b).

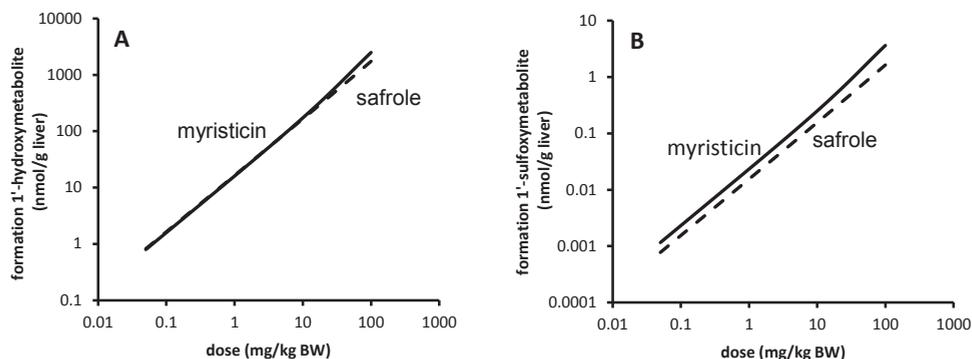


Figure 10. PBK-predicted dose-dependent formation (nmol / g liver) of (A) 1'-hydroxymetabolites and (B) 1'-sulfoxymetabolites of myristicin (—) and safrole (---) in male rat liver.

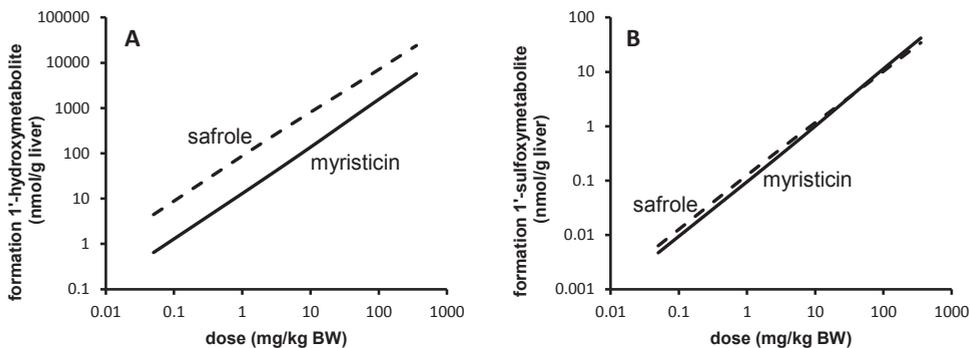


Figure 11. PBK-predicted dose-dependent formation (nmol / g liver) of (A) 1'-hydroxymetabolites and (B) 1'-sulfoxymetabolites of myristicin (—), and safrole (---) in human liver.

Implications for risk assessment

The margin-of-exposure (MOE) concept was applied to assess the possible risks for human health resulting from the daily exposure to myristicin (JECFA 2005; EFSA 2005; Barlow et al. 2006; O'Brien et al. 2006). The MOE is a dimensionless ratio based on a reference point

representing a dose causing a low but measurable cancer incidence in experimental animals (e.g., a BMDL₁₀), which is divided by the estimated daily intake in humans (EFSA 2005). When the MOE is lower than 10000, the compound of interest is considered to be a priority for risk management actions and a concern for human health (EFSA 2005). To date, tumor data for myristicin, from which a BMDL₁₀ can be derived, are absent in the available literature, hampering the application of the MOE approach in the risk assessment of myristicin. Nevertheless, the results of the PBK model predictions presented above indicate that at dose levels in the range of the BMDL₁₀ values, the formation of 1'-sulfoxy metabolites of myristicin in human liver is comparable (1.1-fold higher) to that of the structurally related safrole. On the basis of these considerations, and the limited difference in 1'-sulfoxy metabolite formation for myristicin and safrole in rat and human liver, it was concluded that the BMDL₁₀ values of safrole could be used to perform an initial MOE-based risk assessment for myristicin. Using the BMDL₁₀ for safrole of 1.9–5.1 mg/kg bw per day (van den Berg et al. 2011) and an estimated daily intake of myristicin of 0.0019 mg/kg bw per day (WHO 2009) from use of herbs and spices, the MOE for myristicin would amount to 1000–2684. The estimated daily intake of safrole from spice and spice oil in the USA amounts to 0.001 mg/kg bw per day (WHO 2009). These data result in an MOE for safrole of 1900–5100, indicating a priority for risk management that is somewhat lower than for myristicin.

DISCUSSION

In the presented paper, the recently developed mode of action-based PBK models for detoxification and bioactivation of the alkenylbenzene safrole (Martati et al. 2011, 2012) in male rat and human were extended to the structurally related alkenylbenzene myristicin. The newly developed PBK models combine biochemical and physicochemical information on myristicin and on the physiology of the organism of interest (i.e., rat and human), enabling

the quantification of detoxification and bioactivation in rat and human at realistic low exposure levels. The development of these models facilitates a risk assessment based on read-across from data on safrole, for which *in vivo* chronic toxicity studies are available, to myristicin, a compound for which toxicity data are limited.

The PBK models for myristicin defined in the present paper were able to predict the overall formation of the reactive 1'-sulfoxymyristicin metabolite in the liver of rat and human, thus enabling comparison to the overall formation of the 1'-sulfoxy metabolites of the corresponding alkenylbenzene safrole. Comparison of the rat and human PBK model predictions indicated an only limited species-dependent difference in the overall metabolic activation of myristicin. The difference observed was within the default factor of 4, which is generally used to describe kinetic differences between species (IPCS 2010). The newly developed PBK model for myristicin was also used to compare the levels of metabolic activation of myristicin to those predicted previously for safrole in male rat and human liver (Martati et al. 2011, 2012). Results reveal that the formation of the proximate and ultimate carcinogenic metabolites of myristicin and safrole in rat liver appears to be comparable varying about 1.4-fold and 2.2-fold, respectively, with rat liver bioactivation of myristicin predicted to be somewhat higher than that of safrole for both metabolites. In humans, the formation of especially the 1'-sulfoxy metabolites of the two alkenylbenzenes is predicted to be comparable (1.1-fold difference). The PBK model outcomes obtained for the formation of reactive 1'-sulfoxy metabolites of myristicin and safrole can be compared to the relative bioactivation of these two alkenylbenzenes observed in other studies. In an *in vitro* study with cultured human cells, the ability of myristicin to form DNA adducts upon exposure of the cells to myristicin appeared to be almost the same as upon exposure to safrole (Zhou et al. 2007b) which is in line with the relative differences predicted by the PBK models. Data derived from another study in which mice were exposed to the alkenylbenzenes via

intraperitoneal (i.p) injection (Table 1) suggest that the DNA adduct formation of safrole and myristicin is also not much different with DNA adduct formation in the liver of safrole being 2.7 times higher than that of myristicin (Randerath et al. 1984). In a parallel study, Phillips et al. (Table 1) reported that in neonatal mice, both safrole and myristicin were able to form DNA adducts in liver, and the DNA binding levels of safrole and myristicin were 17.5 and 7.8 pmol/mg DNA, respectively (Phillips et al. 1984). It is important to note that in these mice studies, DNA adduct levels were quantified by ^{32}P post-labeling which is known to be less accurate than, for example, LC-MS in the quantification of DNA adduct levels (Randerath et al. 1984). On the basis of the results now available, it can be concluded that using data on safrole for a read-across to myristicin is a reasonable approach for an initial risk assessment on myristicin. Such a risk assessment for myristicin can be based on the MOE approach. Because data on tumor formation are currently not available for myristicin, risk assessment for myristicin was performed using the BMDL_{10} values for tumor formation by safrole, given the comparable bioactivation in human liver predicted by the newly developed PBK models, where the difference in the bioactivation of myristicin and safrole was predicted to be only 1.1-fold.

Using the BMDL_{10} for safrole of 1.9–5.1 mg/kg bw per day (van den Berg et al. 2011) and an estimated daily intake of myristicin of 0.0019 mg/kg bw per day (WHO 2009) from use of herbs and spices, the MOE for myristicin would amount to 1000–2684. For comparison, the MOE values of safrole can be given, obtained at the estimated daily intake of safrole from spice and spice oil in the USA that amounts to 0.001 mg/kg bw per day (WHO 2009) and a BMDL_{10} of safrole of 1.9–5.1 mg/kg bw per day (van den Berg et al. 2011). These data result in an MOE for safrole of 1900–5100, indicating a priority for risk management that is lower than for myristicin.

Altogether, the results obtained indicate that PBK modeling provides an important insight into the limited species differences between male rat and human in the metabolic activation of myristicin, and that in human liver, formation of the ultimate carcinogenic 1'-sulfoxy metabolites is almost the same for myristicin and safrole supporting a possibility for the risk assessment for myristicin based on the MOE approach using the BMDL₁₀ for tumor formation of safrole as a reasonable but careful approximation. The present study provides an example of how PBK modeling can facilitate a read-across in risk assessment from compounds for which *in vivo* toxicity studies are available to a compound for which only limited toxicity data have been described, thus contributing to the development of alternatives for animal testing.

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CONFLICT OF INTEREST

Prof. IMCM Rietjens declares she is a member of the Expert Panel of the Flavour and Extract Manufacturers Association (FEMA). Other authors declare that no conflict of interest exists.

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Supplementary material

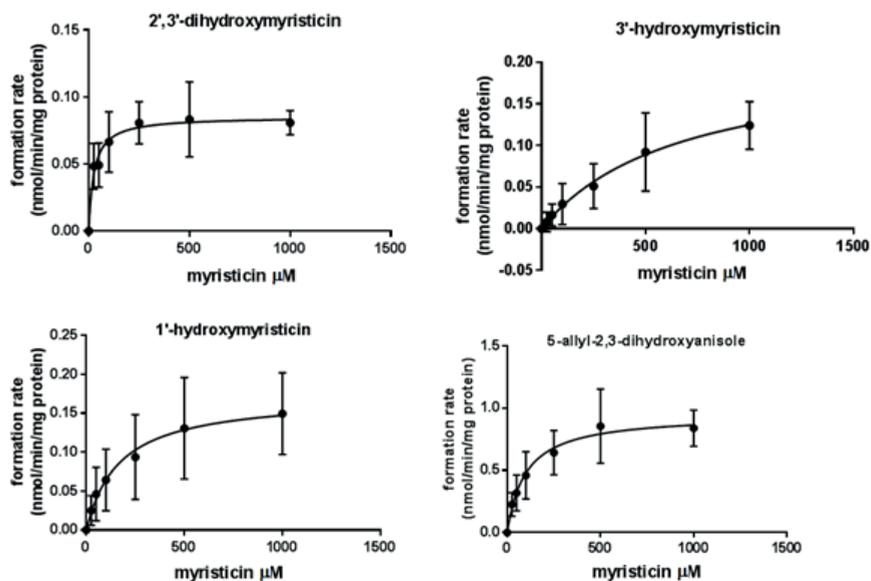


Figure S1. Concentration-dependent rate of myristicin metabolite formation in incubations with pooled human mixed gender liver microsomes. Curves for formation of four different phase I metabolites (2',3'-dihydroxymyristicin, 3'-hydroxymyristicin, 1'-hydroxymyristicin and 5-allyl-2,3-dihydroxyanisole) are presented.



Chapter 3

Mode of action based risk assessment of the botanical food-borne alkenylbenzenes apiol from parsley using physiologically based kinetic (PBK) modelling and read-across from safrole

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ABSTRACT

The present study developed physiologically-based kinetic (PBK) models for the alkenylbenzene apiol in order to facilitate risk assessment based on read-across from the related alkenylbenzene safrole. Model predictions indicate that in rat liver the formation of the 1'-sulfoxy metabolite is about 3 times lower for apiol than for safrole. These data support that the lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence (BMDL₁₀) that would be obtained in a rodent carcinogenicity study with apiol may be 3-fold higher for apiol than for safrole. These results enable a preliminary risk assessment for apiol, for which tumor data are not available, using a BMDL₁₀ value of 3 times the BMDL₁₀ for safrole. Based on an estimated BMDL₁₀ for apiol of 5.7-15.3 mg/kg body wt per day and an estimated daily intake of 4×10^{-5} mg/kg body wt per day, the margin of exposure (MOE) would amount to 140000-385000. This indicates a low priority for risk management. The present study shows how PBK modelling can contribute to the development of alternatives for animal testing, facilitating read-across from compounds for which *in vivo* toxicity studies on tumor formation are available to compounds for which these data are unavailable.

INTRODUCTION

Apiol (1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene) is one of alkenylbenzene compounds found in herbs and other botanicals like parsley, fennel, nutmeg and elemi tree. High levels of apiol are especially present in parsley and apiol gives parsley its characteristic odour (Miller et al., 1983, Parthasarathy, 2008, Tunali et al., 1999). Parsley parts (leaves, stems, roots and seeds) are used as food flavouring agent in many food types such as sauces, soups, stuffing, rissoles, and minces, and are also sprinkled over vegetables or salads. Beside the use of parsley in food, medicinal uses of apiol extracted from the Triple Moss curled variety of parsley have been reported (Parthasarathy, 2008). The structure of apiol is closely related to that of safrole, myristicin, estragole, methyleugenol, and elemicin (Figure 1).

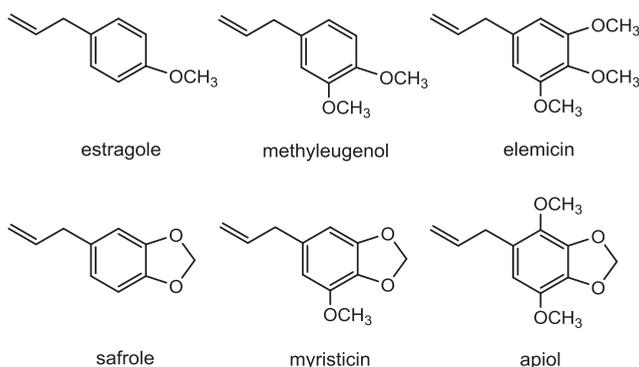


Figure 1. The structural formulas of the alkenylbenzenes estragole, methyleugenol, elemicin, safrole, myristicin and apiol.

These alkenylbenzenes are of concern because they can be converted to DNA reactive 1'-sulfoxy metabolites that readily form DNA adducts that may contribute to the formation of hepatomas in rodent bioassays upon exposure to these alkenylbenzenes at high dose levels (Miller et al., 1983, Randerath et al., 1984, SCF, 2001b, SCF, 2001a, SCF, 2002, Wiseman et al., 1987). DNA adduct formation results from bioactivation of apiol following the

metabolic pathways presented in Figure 2 (WHO, 2009). Apiol however is less well studied than its related alkenylbenzene safrole and rodent carcinogenicity studies on apiol are not available hampering its risk assessment.

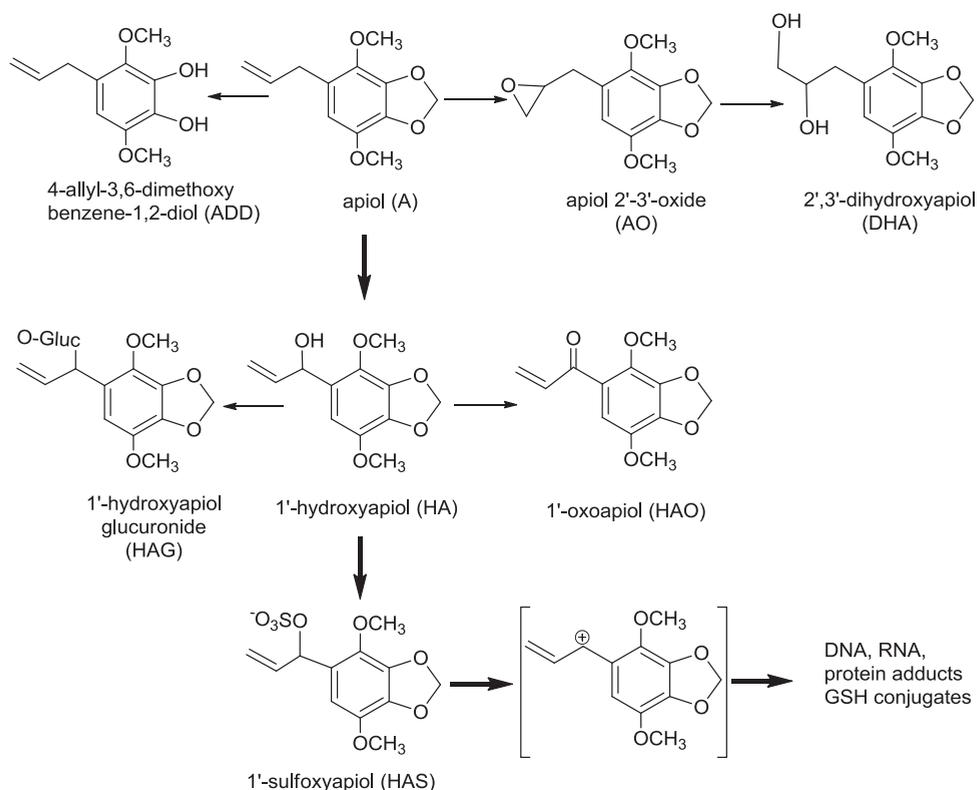


Figure 2. Suggested metabolic pathways of apiol.

The structural similarity of apiol to safrole raises a concern and provides a rationale for an updated risk assessment of exposure to apiol via food including food supplements. Several studies have reported on the DNA adduct formation by apiol in animal *in vivo* studies or in human *in vitro* models as compared to the related alkenylbenzenes and found apiol to be less potent than its structural analogues (Miller et al., 1983, Phillips et al., 1984, Randerath et al., 1984, Zhou et al., 2007).

The safety of human exposure to apiol has been assessed by the WHO in 2009 concluding that the structural similarities to safrole with available experimental data give evidence that apiol may cause toxicity and carcinogenicity in rodents exposed to high dose levels (WHO, 2009). Because rodent bioassays characterizing the possible carcinogenicity of apiol are not available, the aim of the present study was to perform a risk assessment for apiol based on PBK-modelling based read-across from safrole for which rodent tumor data are available (Miller et al., 1983, Phillips et al., 1984, Randerath et al., 1984).

MATERIALS AND METHODS

Chemicals and Reagents:

Caution: Apiol and 1²-hydroxyapiol are possible carcinogens and should be handled with care.

Apiol, uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA), reduced L-glutathione (GSH), alamethicin (from *Trichoderma viride*), and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) were obtained from Sigma Aldrich (Zwijndrecht, Netherlands). Reduced β -nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NAD⁺) were from Roche Diagnostic (Mannheim, Germany), and dimethyl sulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium). Chromatographic grade trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate trihydrate, acetic acid, and magnesium chloride were provided by VWR International (Darmstadt, Germany). Acetonitrile (ACN) (ULC/MS grade) was purchased from Biosolve BV (Valkenswaard, The Netherlands).

Pooled male rat liver microsomes and S9 from Sprague–Dawley and mixed gender pooled human liver microsomes and S9 were from BD Gentest (Woburn, United States). Pooled

male Sprague–Dawley rat lung, kidney, and small intestinal microsomes and pooled gender human lung, kidney, and intestinal microsomes were purchased from BioPredic International (Rennes, France).

Synthesis of 1'-hydroxyapiol and 1'-oxoapiol

The synthesis of 1'-hydroxyapiol from apiol was done as described previously for the synthesis of 1'-hydroxysafrole from safrole by Jeurissen et al. (Jeurissen et al., 2004) and 1'-oxoapiol was synthesized from 1'-hydroxyapiol according to the method used for synthesis of 1'-oxoestragole from 1'-hydroxyestragole (Wislocki et al., 1976).

In vitro incubations

Microsomal metabolism of apiol by rat and human microsomes

Liver, kidney, lung and small intestine microsomes from male Sprague Dawley rat and from human were used to determine tissue-specificity of the microsomal biotransformation of apiol in rat and human. Incubations were performed by adding 1 mg/ml of the microsomal protein preparation to an incubation mixture containing (final concentrations) 3 mM NADPH in 0.2 M Tris-HCl (pH 7.4). After a 1 minute pre-incubation at 37°C, apiol (final concentration 1000 µM) was added from a 100 times concentrated stock solution in DMSO so that the final DMSO content was 1 % (v/v). After 120 minutes incubation at 37°C for rat and human microsomes the reactions were terminated by adding 25 µl ice-cold acetonitrile. All samples were centrifuged for 5 minutes at 16000×g and the supernatant was stored at -20°C until Ultra Performance Liquid Chromatography (UPLC) analysis.

For both male rat and human, metabolism of apiol was only observed in incubations with liver microsomes (See the results section) and hence kinetic constants for the formation of microsomal metabolites were determined only for liver microsomes. To determine the kinetic

constants incubations following the conditions described above were done using final concentrations of apiol from 25 to 1000 μM for rat and human liver while keeping the DMSO content at 1 % (v/v). Blank incubations were performed in the absence of the cofactor NADPH. All incubations were performed in triplicate.

Glucuronidation of 1'-hydroxyapiol to 1'-hydroxyapiol glucuronide by rat and human liver S9

The kinetic constants for the metabolic conversion of 1'-hydroxyapiol to 1'-hydroxyapiol glucuronide in incubations with both male rat and human liver fractions were determined as described previously for related 1'-hydroxyalkenylbenzenes (Al-Subeihi et al., 2012, Al-Subeihi et al., 2011, Martati et al., 2011b, Martati et al., 2012b, Punt et al., 2008, Punt et al., 2009, Van den Berg et al., 2012). In short, incubations were performed using (final concentrations) 10 mM UDPGA and 0.5 mg/mL male Sprague–Dawley rat or pooled gender human S9 protein in 0.2 M Tris-HCl (pH 7.4) containing 10 mM MgCl_2 . First the incubations were pre-treated on ice for 15 minutes with 0.025 mg/mL alamethicin added from a 200 times concentrated stock solution in methanol to overcome enzyme latency and to obtain maximal glucuronidation activity. After the pre-treatment on ice and pre-incubation at 37°C for 1 minute, the reactions were subsequently started by adding 1'-hydroxyapiol in final concentrations of 10 to 2000 μM . 1'-Hydroxyapiol was added from 100 times concentrated stock solutions dissolved in DMSO. The reaction was incubated for 30 and 120 minutes for rat and human S9, respectively, and terminated by adding 25 μL of ice-cold acetonitrile. Blank incubations were carried out in the absence of the cofactor UDPGA. Experiments were performed in triplicate. All samples were centrifuged for 5 minutes at 16000 \times g, and the supernatant was stored at -20°C until UPLC analysis.

Oxidation of 1'-hydroxyapiol to 1'-oxoapiol by rat and human liver microsomes

The kinetic constants for the enzymatic conversion of 1'-hydroxyapiol to 1'-oxoapiol were determined using incubation mixtures containing (final concentrations) 3 mM NAD⁺, 2 mM GSH, and 1 mg/mL rat or human liver microsomes in 0.2 M Tris-HCl (pH 7.4). GSH was added to the incubation mixtures to trap the reactive 1'-oxometabolite formed after oxidation of 1'-hydroxyapiol. To determine the kinetic constants incubations following the conditions described above were done using final concentrations of 1'-hydroxyapiol from 50 to 2000 μM added to the incubation mixture from 100 times concentrated stock solutions in DMSO. After pre-incubation for 1 minute at 37°C the reactions were started by adding the 1'-hydroxyapiol and were terminated after 60 minutes of incubation at 37°C by adding 25 μL of ice-cold acetonitrile. The formation of the GSH conjugate of 1'-oxoapiol, GS-1'-oxoapiol, was linear with time and microsomal protein under the experimental conditions used. Blank incubations were performed without the cofactor NAD⁺. Incubations were performed in triplicate. All samples were centrifuged for 5 minutes at 16000×g, and the supernatant was stored at -20°C until UPLC analysis.

Sulfonation of 1'-hydroxyapiol to 1'-sulfoxyapiol by rat and human liver S9

The kinetic constants for the enzymatic conversion of 1'-hydroxyapiol to 1'-sulfoxyapiol were determined using incubation mixtures containing (final concentrations) 10 mM GSH, 0.2 mM PAPS, and 3 mg/mL Sprague–Dawley rat or mixed gender pooled human liver S9 proteins in 0.1 M potassium phosphate buffer (pH 8.0). GSH was used as a scavenger of the reactive carbocation formed due to the unstable nature of the 1'-sulfoxymetabolite in an aqueous environment. After pre-incubation for 1 minute at 37°C 1'-hydroxyapiol dissolved in DMSO was added in final concentrations ranging between 50 and 2000 μM, while keeping the final DMSO content at 1 % (v/v). The reaction was terminated after 360 minutes by

adding 25 μ L of ice-cold acetonitrile. The formation of the GSH conjugate of 1'-sulfoxyapiol was linear with time and S9 protein concentration under the experimental conditions used. The scavenging may either be chemically or catalysed by the glutathione S-transferases present in the S9 incubations in which the sulfonation of 1'-hydroxyapiol was measured. Blank incubations were performed in the absence of PAPS. Incubations were performed in triplicate. All samples were centrifuged for 5 minutes at 16000 \times g, and the supernatant was stored at -20°C until UPLC analysis.

Identification and quantification of apiol metabolites

Apiol metabolites formed in microsomal incubations were identified using a photodiode array detection (UPLC-PDA) composed of a Waters (Waters, Milford, MA) Acquity solvent manager, sample manager, and diode array detector, equipped with a Water Acquity UPLC BEH C18 column. The gradient was made with a mixture of ACN and ultrapure water containing 0.1 % (v/v) TFA. The flow rate was 0.6 ml/min. After equilibrating the column at the starting condition of 10 % ACN, ACN increased to 50 % over 4 min, was further increased to 80 % over 0.5 min, decreased to 10 % over 0.5 min and was finally kept at 10 % for 1 min. Metabolites were identified as described in the Result section. Quantification of microsomal apiol metabolites was done by comparison of the peak areas obtained at a wavelength of 208 nm to the calibration curve of synthesized 1'-hydroxyapiol because UV spectra of all metabolites were similar.

The concentration of 1'-hydroxyapiol glucuronide was determined using the same UPLC-PDA system as described above. The gradient was made with acetonitrile and ultrapure water containing 0.1 % (v/v) TFA. The flow rate was 0.6 ml/min. In a mixture of ACN and ultrapure water containing 0.1 % (v/v) TFA, the gradient started at 10 % ACN, increased to 60% ACN over 3.5 minutes, further increased to 80% over 0.5 min, kept at 80% ACN for 0.5

min and then decreased to 10 % ACN over 0.5 min. 1'-hydroxyapiol glucuronide was identified as described in the results section. Quantification of 1'-hydroxyapiol glucuronide was done by comparison of the peak areas obtained at a wavelength of 208 nm to the calibration curve of synthesized 1'-hydroxyapiol.

The concentration of the glutathione conjugate of 1'-oxoapiol was determined using the same UPLC-PDA system as described above. The gradient was made with acetonitrile and ultrapure water containing 0.1 % (v/v) TFA. The flow rate was 0.6 ml/min. In a mixture of ACN and ultrapure water containing 0.1 % (v/v) TFA, the gradient started at 10% ACN, increased to 30 % ACN over 2.5 minutes, was further increased to 80 % ACN over 0.5 min, kept at 80 % ACN for 0.5 min and finally decreased to 10 % ACN over 0.5 min, and kept at 10 % ACN for 0.5 min. Quantification of the GSH adduct of the metabolite was done by comparison of the peak areas obtained at a wavelength of 280 nm to the calibration curve of the GSH adduct of the synthesized 1'-oxoapiol.

The concentration of the glutathione conjugate of 1'-sulfoxyapiol was determined using the same UPLC-PDA system described above. The flow rate was 0.6 ml/min. In a mixture of ACN and ultrapure water containing 0.1 % (v/v) TFA, the gradient started at 0 % ACN, increased to 20 % ACN over 0.2 minutes, further increased to 30 % ACN over 4.3 min, and again increased to 100 % ACN over 0.3 min, kept at 100 % ACN for 0.2 min, and finally decreased to 0 % ACN over 0.2 min and kept at 0 % ACN for 0.8 min. The GSH adduct of 1'-sulfoxyapiol formed was identified by LC-MS using a microTOF MS (Bruker) coupled to an Agilent LC (1200 Series) equipped with an Altima C18 column (150 x 4.6 mm, 3 µm). The mobile phase used consisted of nanopure water with 0.1 % formic acid and ACN with 0.1 % formic acid. Elution was at a flow rate of 0.8 ml/min, starting at 22 % ACN with a linear increase to 100 % ACN in 30 min. Subsequently, the gradient returned linearly to the initial condition in 2 min and remained 13 min at this condition prior to the next injection.

Quantification of the GSH adduct of 1'-sulfoxyapiol was done by comparison of the peak areas obtained at a wavelength of 280 nm to the calibration curve of the GSH adduct of synthesized 1'-oxoapiol since their UV spectra are comparable. Mass spectrometric analysis was in the negative electrospray mode using a spray capillary voltage of 4500 V, a capillary temperature of 200°C and nitrogen as nebulizer gas at 8.0 L/min.

Determination of kinetic constants of apiol and 1'-hydroxyapiol metabolic conversions

Kinetic constants for the metabolic conversions of apiol and 1'-hydroxyapiol were calculated by two equations depending on the dose-dependency of the kinetics of the conversion. If the reaction reached saturation with increasing substrate concentration, the kinetic constants were derived by fitting the data to the standard Michaelis-Menten equation; $v = V_{\max} \times [S]/(K_m + [S])$, in which [S] represents the substrate concentration, V_{\max} the maximum velocity and K_m the Michaelis-Menten constant for the formation of the different metabolites of apiol or 1'-hydroxyapiol. Data analysis was accomplished using GraphPad Prism, version 5.04 (GraphPad Software, San Diego, California, USA). When the reaction did not reach saturation and rather displayed a linear increase in the rate of conversion with increasing substrate concentration, the kinetic rate constant for a first order reaction was derived by using the following equation; $v = k \cdot [S]$, with k being the first order rate constant and [S] representing the substrate concentration.

Apiol PBK models in rat and human

PBK models were developed describing the dose-dependent bioactivation and detoxification of apiol in respectively rat and human. The apiol models were based on the PBK models previously defined for the metabolism of estragole, methyleugenol, elemicin and safrole in rat and human (Al-Subeihi et al., 2011, Al-Subeihi et al., 2012, Martati et al., 2011, Martati et

al., 2012, Punt et al., 2008, Punt et al., 2009). Figure 3 presents an overview of the developed PBK models for apiol metabolism in rat and human.

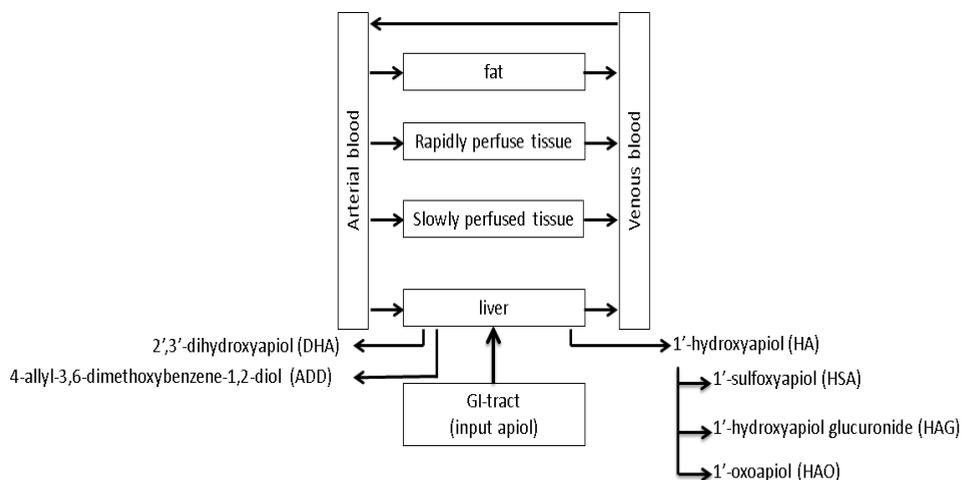


Figure 3. Overview of the PBK model for apiol.

The models consist of several compartments representing different organs and tissues (*i.e.* liver, fat tissue, rapidly perfused tissues and slowly perfused tissues) which are connected through the systemic circulation. First order kinetics was used to describe the uptake of apiol from the gastrointestinal tract assuming a direct and complete uptake by the liver with an absorption rate constant (k_a) of 1.0 h^{-1} which is based on the fast and complete absorption of the structurally related alkenylbenzene estragole from the gastrointestinal tract (WHO, 2009). For both rat and human, only the liver microsomes were able to convert apiol to different microsomal metabolites (see results section) and this is why no other tissue compartments were described as separate compartments in the model. In liver microsomal incubations 2',3'-dihydroxyapiol (DHA), 1'-hydroxyapiol (HA), and 4-allyl-3,6-dimethoxybenzene-1,2-diol (ADD) were formed and their formation was included. Since 2',3'-dihydroxyapiol is formed after hydrolysis of apiol-2',3'-oxide, data for both metabolites that result from epoxidation of

apiol were combined and referred to as 2',3'-dihydroxyapiol. Accordingly, a mass balance equation for apiol in rat and human liver is as follows:

$$\begin{aligned} \partial AL_A / \partial t &= \partial Uptake_A / \partial t + QL \times (CA_A - CL_A / PL_A) \\ &\quad - V_{max,HA} \times CL_A / PL_A / (K_{m,HA} + CL_A / PL_A) \\ &\quad - V_{max,DHA} \times CL_A / PL_A / (K_{m,DHA} + CL_A / PL_A) \\ &\quad - V_{max,ADD} \times CL_A / PL_A / (K_{m,ADD} + CL_A / PL_A) \\ \partial Uptake / \partial t &= -\partial AGI_A / \partial t = K_a \times AGI_A \\ AGI_A(0) &= \text{oral dose} \\ CL_A &= AL_A / VL \end{aligned}$$

Where uptake_A (μmol) is the amount of apiol taken up from the gastrointestinal tract, AGI_A (μmol) is the amount of apiol remaining in the gastrointestinal tract, AL_A (μmol) is the amount of apiol in liver. CL_A is the apiol concentration in the liver (μmol/L). CA_A is the apiol concentrations in the arterial blood (in μmol/L), QL is the blood flow rate to liver (L/h), VL is the volume of the liver (L), PL_A is the liver/blood partition coefficient of apiol, and V_{max}, and K_m, are the values representing the maximum rate of formation and Michaelis-Menten constant, respectively, for the formation of 1'-hydroxyapiol (HA), 2',3'-dihydroxyapiol (DHA), and 4-allyl-3,6-dimethoxybenzene-1,2-diol (ADD).

In both models, the conversion of apiol to 2',3'-dihydroxyapiol and 4-allyl-3,6-dimethoxybenzene-1,2-diol are described, but further reactions with these metabolites were not included in the model. It was assumed that these metabolites and/or their possible conjugates were completely excreted into the urine. Since the liver is the major target organ for alkenylbenzene-induced carcinogenicity in rats and mice, the present study focused primarily on metabolic conversion of 1'-hydroxyapiol by glucuronidation, oxidation and sulfonation in the liver only. The mass balance equation for 1'-hydroxyapiol in rat and human liver is as follows:

Rat:

$$\begin{aligned} \partial AL_{HA}/\partial t &= V_{max,HA} \times CL_A/PL_A / (K_{m,HA} + CL_A/PL_A) \\ &\quad - V_{max,HAG} \times CL_{HA}/PL_{HA} / (K_{m,HAG} + CL_{HA}/PL_{HA}) \\ &\quad - k_{HAO} \times CL_{HA}/PL_{HA} \\ &\quad - k_{HAS} \times CL_{HA}/PL_{HA} \\ CL_{HA} &= AL_{HA}/VL \end{aligned}$$

Human:

$$\begin{aligned} \partial AL_{HA}/\partial t &= V_{max,HA} \times CL_A/PL_A / (K_{m,HA} + CL_A/PL_A) \\ &\quad - V_{max,HAG} \times CL_{HA}/PL_{HA} / (K_{m,HAG} + CL_{HA}/PL_{HA}) \\ &\quad - V_{max,HAO} \times CL_{HA}/PL_{HA} / (K_{m,HAO} + CL_{HA}/PL_{HA}) \\ &\quad - k_{HAS} \times CL_{HA}/PL_{HA} \\ CL_{HA} &= AL_{HA}/VL \end{aligned}$$

where AL_{HA} is the amount of 1'-hydroxyapiol in the liver (μmol), CL_{HA} is the 1'-hydroxyapiol concentration in the liver ($\mu\text{mol/L}$), PL_{HA} is the liver/blood partition coefficient of 1'-hydroxyapiol, V_{max} , and K_m , are the maximum rate of formation and Michaelis-Menten constant, respectively, for the formation of the different apiol metabolites, including 1'-hydroxyapiol glucuronide (HAG) in the rat and human liver and 1'-oxoapiol (HAO) in human, and k is the first order rate constant for formation of 1'-oxoapiol in rat, and 1'-sulfoxyapiol (HAS) in rat and human liver.

V_{max} and K_m values and first order rate constants k in case of absence of saturation (for oxidation in rat and sulfonation of 1'-hydroxyapiol) for the different metabolic pathways of apiol and 1'-hydroxyapiol were derived *in vitro* in the present study. V_{max} values that were derived *in vitro* expressed as $\text{nmol min}^{-1} (\text{mg liver microsomal or S9 protein})^{-1}$ were scaled to values representing the V_{max} per $\mu\text{mol h}^{-1} (\text{g liver})^{-1}$ using microsomal yields of 35 mg/g liver in rat and 32 mg/g liver in human and S9 protein yields of 143 mg/g liver as defined by Punt

et al. (Punt et al., 2008, Punt et al., 2009) based on Medinsky *et al.* (Medinsky et al., 1994). First order rate constants k expressed in ml min^{-1} ($\text{mg liver microsomal or S9 protein}$)⁻¹ were scaled to values expressed in ml h^{-1} per g liver using the same conversion factor for microsomal and S9 protein yield.

Tables 1 and 2 summarize the physiological parameters (*i.e.* tissue volumes, cardiac output and tissue blood flows) for rat and human respectively which were derived from literature (Brown et al., 1997). Partition coefficients were derived *in silico* based on a method described by DeJongh *et al.* (DeJongh et al., 1997) using the $\log K_{ow}$. $\log K_{ow}$ values for apiol (ClogP 3.12) and 1'-hydroxyapiol (ClogP 1.56) were estimated using ChemBio3D 2010 (CambridgeSoft, USA). Mass balance equations were coded and numerically integrated in Berkeley Madonna 8.3.18 (Macey and Oster, UC Berkeley, CA, USA) using the Rosenbrock's algorithm for stiff systems. The PBK models were run during 720-hr after the dosing to allow full conversion of the parent compound.

Table 1. Parameters used in the physiologically based biokinetic model for apiol in male rat.

Physiological parameters		Tissue: blood partition coefficients	
Body wt (kg)	0.25		
Percentage of body wt		apiol	
liver	3.4	liver	2.40
fat	7.0	fat	75.93
rapidly perfused	5.1	rapidly perfused	2.40
slowly perfused	60.2	slowly perfused	0.80
arterial blood	1.85		
venous blood	5.55		
Cardiac output (L/h)	5.4	1'-hydroxyapiol	
Percentage of cardiac output		liver	1.10
liver	25.0		
fat	7.0		
rapidly perfused	51.0		
slowly perfused	17.0		

Table 2. Parameters used in the physiologically based biokinetic model for apiol in human.

Physiological parameters		Tissue: blood partition coefficients	
Body wt (kg)	60		
Percentage of body wt		apiol	
liver	2.6	liver	6.45
fat	21.4	fat	104.74
rapidly perfused	5.0	rapidly perfused	6.45
slowly perfused	51.7	slowly perfused	4.08
blood	7.9		
		1'-hydroxyapiol	
Cardiac output (L/h)	310	liver	1.55
Percentage of cardiac output			
liver	22.7		
fat	5.2		
rapidly perfused	47.3		
slowly perfused	24.8		

Model sensitivity analysis using normalized sensitivity coefficients

A sensitivity analysis was used to determine which parameters have the greatest influence on model predictions, and performed as described previously (Al-Subeihi et al., 2012, Al-Subeihi et al., 2011, Martati et al., 2011, Martati et al., 2012, Punt et al., 2008, Punt et al., 2009, Van den Berg et al., 2012). For this purpose normalized sensitivity coefficients (SC) were determined using the following equation: $SC = (C' - C)/(P' - P) \cdot (P/C)$, with C being the initial value of model output, C' the modified value of the model output resulting from an increase in parameter value, P being the initial parameter value and P' representing the modified parameter value. An increase of 5 % in parameter values was used to analyse the effect of a change in parameter on the formation of 1'-hydroxyapiol and 1'-sulfoxyapiol

(expressed as a percentage of the dose over 720-hr). Each parameter was analysed individually while the other parameters were kept at their initial value.

Comparison of the newly developed apiol PBK models based predictions with PBK model predictions for the structurally related compound safrole

The PBK model-based predictions for the formation of 1'-hydroxyapiol and 1'-sulfoxyapiol were compared to the PBK model-based predictions for the dose-dependent formation of the 1'-hydroxy and 1'-sulfoxymetabolites of the structurally related alkenylbenzene safrole. For this purpose the previously defined PBK models for safrole in rat and human described by Martati *et al.* (Martati et al., 2011, Martati et al., 2012) were used. For comparison with apiol, the models describing the metabolism of safrole were also run for 720-hr.

RESULTS

To identify the organs involved in the metabolism of apiol in both male rat and human, incubations were performed using microsomal protein preparations from liver, kidney, lung and small intestine. UPLC analysis of these incubations revealed that for both rat and human conversion of apiol was detected only in incubations with liver microsomes. An example of a chromatogram of an incubation of apiol with male rat liver microsomes and NADPH as the cofactor was shown in Figure 4.

The metabolites formed in incubations with both rat and human liver microsomes were 2',3'-dihydroxyapiol (RT 1.5 minutes), 1'-hydroxyapiol (RT 2.4 minutes), and 4-allyl-3,6-dimethoxybenzene-1,2-diol (RT 2.2 minutes). Identification of 1'-hydroxyapiol was done based on comparison of the UV-spectrum and retention time of the formed 1'-hydroxyapiol with those of the specific synthesized reference compound.

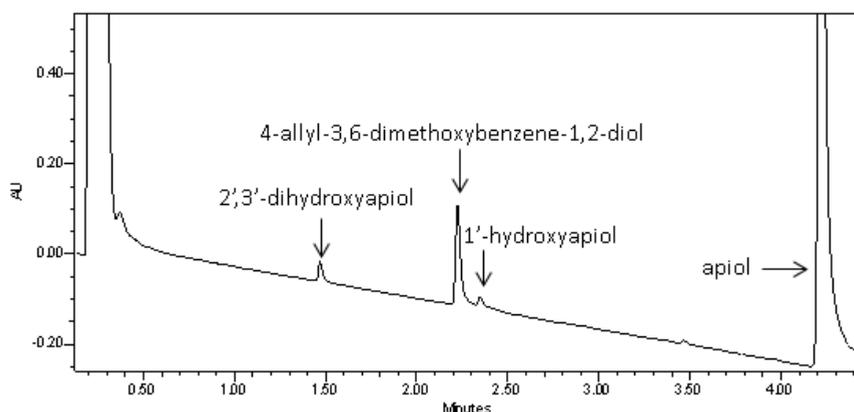


Figure 4. Chromatogram showing the different metabolites of apiol formed in incubations with male rat liver microsomes.

Tentative identification of 4-allyl-3,6-dimethoxybenzene-1,2-diol, resulting from the *O*-demethylation of apiol (Benedetti et al., 1977, Beyer et al., 2006, Lee et al., 1998), and 2',3'-dihydroxyapiol was based on analogy to UPLC chromatograms of metabolic conversions of safrole and the fact that apiol-2',3'-oxide, formed following incubation of apiol with liver microsomes and NADPH, would be hydrolysed by epoxide hydrolase to 2',3'-dihydroxyapiol as was previously demonstrated for the structurally related alkenylbenzenes (Al-Subeihi et al., 2011, Martati et al., 2011, Punt et al., 2008, Luo et al., 1992, Luo and Guenther, 1996, Guenther and Luo, 2001). Furthermore the formation of these three major metabolites is in line with the overview presented by JECFA of the three primary biotransformation pathways for methylenedioxy-substituted alkenylbenzenes including safrole, myristicin and apiol (WHO, 2009). Safrole, myristicin and apiol were reported to be converted primarily by 1,2 ring-*O*-demethenylation yielding diphenolic derivatives (Benedetti et al., 1977, Beyer et al., 2006, Lee et al., 1998), by epoxidation of the double bond in the allyl side-chain yielding the 2',3'-epoxide (Luo and Guenther, 1996, Luo et al., 1992), and by hydroxylation of the alkene side-chain to yield the 1'-hydroxy

metabolite (Benedetti et al., 1977, Miller et al., 1983, Zangouras et al., 1981). Apiol concentration-dependent formation of the various microsomal metabolites was also characterised and the kinetic constants derived from these data on the concentration-dependent microsomal metabolite formation of apiol in incubations with both male rat and human liver samples are presented in Table 3.

Glucuronidation of 1'-hydroxyapiol to 1'-hydroxyapiol glucuronide by rat and human liver S9

UPLC analysis of incubations with male rat liver S9 or mixed gender pooled human liver S9, UDPGA as cofactor and 1'-hydroxyapiol as substrate revealed a peak at 1.5 minutes, representing 1'-hydroxyapiol glucuronide since this peak was increased with increasing apiol concentration. Chromatographic analysis of blank incubations performed in the absence of the cofactor UDPGA did not show this peak at a retention time of 1.50 minutes, and the formation and elution behaviour of the metabolite was in analogous to results from similar incubations with safrole (Martati et al., 2011, Martati et al., 2012).

The 1'-hydroxyapiol concentration-dependent increase in glucuronidation of 1'-hydroxyapiol to 1'-hydroxyapiol glucuronide in incubations with both male rat and human liver fractions is presented in figures 5A and 5D respectively. The kinetic constants derived from these data are presented in Table 3.

Table 3. Kinetic constants for metabolism of apiol and 1'-hydroxyapiol in incubations with Sprague-Dawley male rat liver microsomes or S9 fractions and mixed gender pooled human liver microsomes or S9 fractions (n=3).

Metabolite	SPECIES				RAT			HUMAN		
	Abbreviation	$K_{m^{a,b}} \pm SD$	$V_{max^{a,c}} \pm SD$	$k^d \pm SD$	<i>in vitro</i> catalytic efficiency ^e	$K_{m^{a,b}} \pm SD$	$V_{max^{a,c}} \pm SD$	$k^d \pm SD$	<i>in vitro</i> catalytic efficiency ^e	
2',3'-dihydroxyapiol	DHA	152 ± 18	0.15 ± 0.01		0.99	67 ± 7	0.032 ± 0.026		0.48	
1'-hydroxyapiol	HA	95 ± 19	0.10 ± 0.02		1.05	102 ± 38	0.035 ± 0.033		0.34	
4-allyl-3,6-dimethoxybenzene-1,2-diol	ADD	37 ± 7.7	0.81 ± 0.07		21.89	137 ± 18	0.58 ± 0.45		4.2	
1'-hydroxyapiol glucuronide	HAG	621 ± 459	5.1 ± 1.8		8.21	381 ± 350	0.28 ± 0.19		0.74	
1'-oxoapiol	HAO					2592 ± 755.7	1.13 ± 0.28		0.44	
1'-sulfoxyapiol	HAS									

^a Mean values of three independent measurements ± SD

^b μM

^c nmol min⁻¹ (mg microsomal or S9 protein)⁻¹

^d First order rate constant for product formation (ml/min/mg protein)

^e μl min⁻¹ (mg microsomal or S9 protein)⁻¹, ($V_{max}/K_m \times 1000$ μl/ml)

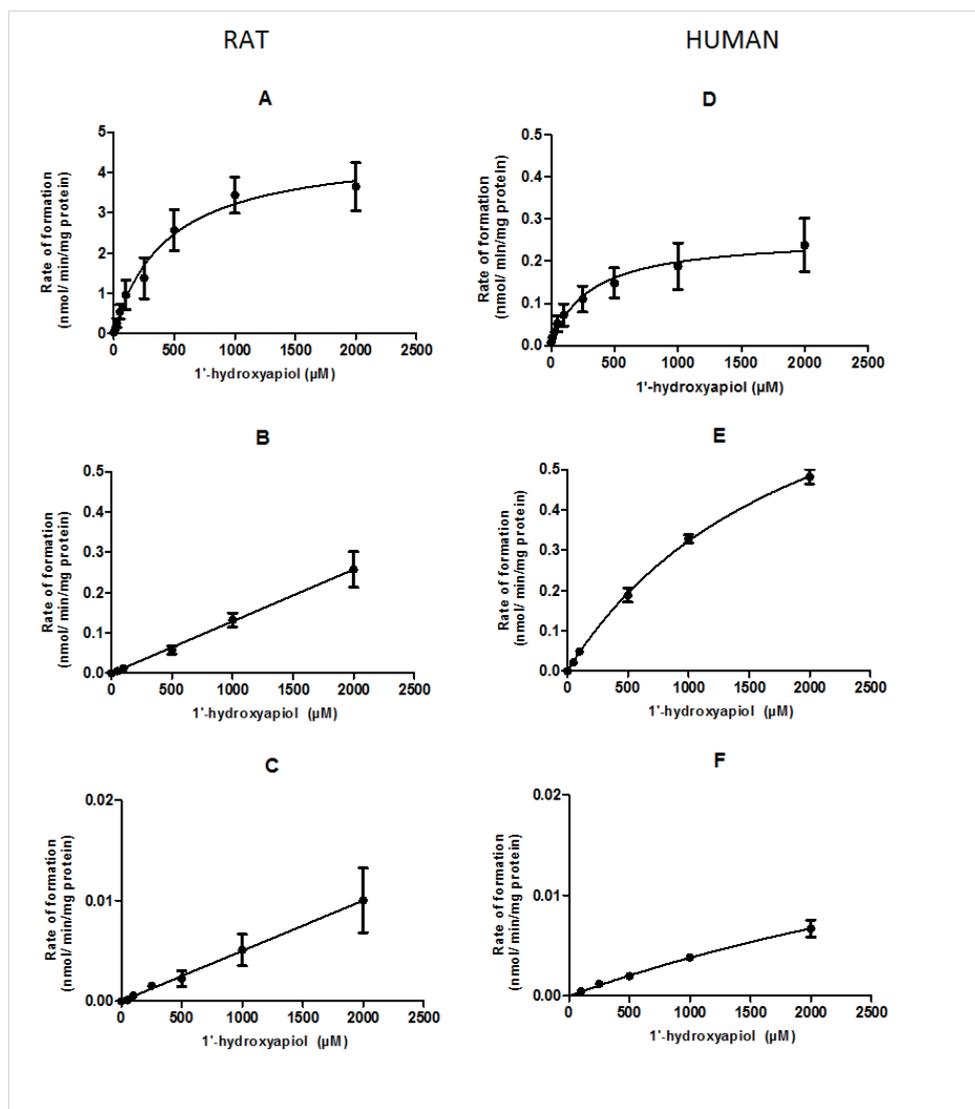


Figure 5. Concentration-dependent rate of (A) glucuronidation of 1'-hydroxyapiol in incubations with pooled male rat liver S9 or (D) pooled human mixed gender liver S9, (B) oxidation of 1'-hydroxyapiol in incubations with pooled male rat liver microsomes or (E) pooled human mixed gender liver S9, and (C) sulfonation of 1'-hydroxyapiol in pooled male rat liver S9 or (F) pooled human mixed gender liver S9. Data points represent mean values \pm SD of three or four individual experiments.

Oxidation of 1'-hydroxyapiol to 1'-oxoapiol by rat and human liver microsomes

UPLC analysis of incubations with male rat liver microsomes or mixed gender pooled human liver microsomes, NAD⁺ as cofactor and 1'-hydroxyapiol as substrate revealed a peak at 2.0 minutes. In this incubation GSH was also added to trap the reactive oxidation metabolite 1'-oxoapiol. The peak at 2.0 minutes was identified as the GSH conjugate of 1'-oxoapiol, GS-1'-oxoapiol. Identification of GS-1'-oxoapiol was done based on comparison of the UV-spectrum and retention time of the formed GS-1'-oxoapiol with those of the synthesized reference compound.

The rate of oxidation of 1'-hydroxyapiol in male rat liver microsomes and pooled human liver microsomes with increasing concentrations of 1'-hydroxyapiol is presented in figures 5B and 5E respectively. Table 3 presents the kinetic constants derived from these data.

Sulfonation of 1'-hydroxyapiol to 1'-sulfoxyapiol by rat and human liver S9

The reactive 1'-sulfoxyapiol formed upon sulfonation of the proximate carcinogenic metabolite 1'-hydroxyapiol was trapped by using GSH. The scavenging may be either chemically or catalysed by the glutathione S-transferases present in the S9 incubations in which the sulfonation of 1'-hydroxyapiol was measured.

UPLC analysis of incubations with male rat liver S9 or mixed gender pooled human liver S9, 1'-hydroxyapiol, PAPS and GSH revealed a peak at 1.3 minutes. The LC-MS analysis showed increased signal of m/z 526 with negative ion mode. The ion at m/z 526 corresponds to the theoretically expected $[M-H]^-$ mass of a GSH adduct of 1'-sulfoxyapiol. Figures 5C and 5F present the 1'-hydroxyapiol concentration dependent rate of formation of 1'-sulfoxyapiol in incubations with male rat liver S9 or mixed gender pooled human liver S9. The kinetic constants for the conversion of 1'-hydroxyapiol to 1'-sulfoxyapiol in rat and human liver derived from these data are presented in Table 3.

Comparison of the kinetic constants for conversion of apiol and 1'-hydroxyapiol by male rat and mixed gender pooled human tissue fractions

In order to allow a comparison between the kinetic constants for metabolism of apiol and 1'-hydroxyapiol by male rat and mixed gender pooled human tissue fractions, V_{\max} values that were derived *in vitro* expressed as $\text{nmol min}^{-1} (\text{mg microsomal or S9 protein})^{-1}$ were scaled to values representing the V_{\max} per $\mu\text{mol h}^{-1} (\text{g tissue})^{-1}$ using microsomal and S9 protein yields as described in literature (Medinsky et al., 1994) and previously used by Punt *et al.*, (Punt et al., 2009, Punt et al., 2008), Al-Subeihi *et al.* (Al-Subeihi et al., 2012, Al-Subeihi et al., 2011) and Martati *et al.* (Martati et al., 2012, Martati et al., 2011). The *in vivo* V_{\max} values derived accordingly, were used to calculate a scaled catalytic efficiency (scaled V_{\max} *in vivo* / K_m) for the formation of apiol metabolites (Table 4). These values revealed that the catalytic efficiency for formation of the proximate carcinogenic metabolite of apiol, 1'-hydroxyapiol, is 3.3 times higher in male rat liver than in human liver. The difference in the formation rate is mainly due to the 3-fold difference in the maximum velocity (V_{\max}) for the conversion of apiol to 1'-hydroxyapiol (see Table 3).

The formation of 1'-hydroxyapiol glucuronide was found to represent the main metabolic reaction for detoxification of 1'-hydroxyapiol in rat and human liver while in rat liver the catalytic efficiency for this conjugation was found to be 11.2-fold higher than in human.

For 1'-hydroxyapiol oxidation, the scaled k for rat liver was found to be 3.1 times lower than the scaled *in vivo* catalytic efficiency for human liver. While for sulfonation the scaled k was 1.3-fold higher in rat liver than in human liver.

Altogether, the results revealed that glucuronidation of 1'-hydroxyapiol, representing a detoxification pathway, is the most important pathway in rat and in human for conversion of 1'-hydroxyapiol, and bioactivation of 1'-hydroxyapiol by sulfonation was found to represent only a minor pathway in both rat and human.

Table 4. Scaled kinetic constants for metabolic conversion of apiol and 1'-hydroxyapiol by male rat and human liver fractions.

species	Metabolite	K_m^a (μ M)	scaled V_{max} <i>in vivo</i> ^b (μ mol/h/g tissue)	<i>in vivo</i> catalytic efficiency ^c (ml/h/g tissue)	$k^d \pm$ SD (ml/min/mg protein)	Scaled k^e (ml/h/g tissue)
RAT	2',3'-dihydroxyapiol	152 \pm 18	0.32	2.07		
	1'-hydroxyapiol	95 \pm 19	0.21	2.21		
	4-allyl-3,6-dimethoxybenzene-1,2-diol	37 \pm 8	1.7	46.00		
HUMAN	1'-hydroxyapiol glucuronide	621 \pm 459	43.76	70.75		
	1'-oxoapiol				$13 \times 10^{-5} \pm 3 \times 10^{-5}$	0.27
	1'-sulfoxyapiol				$61 \times 10^{-7} \pm 15 \times 10^{-7}$	0.052
HUMAN	2',3'-dihydroxyapiol	67 \pm 7	0.061	0.92		
	1'-hydroxyapiol	102 \pm 38	0.67	0.66		
	4-allyl-3,6-dimethoxybenzene-1,2-diol	138 \pm 18	1.11	8.13		
HUMAN	1'-hydroxyapiol glucuronide	381 \pm 350	2.40	6.31		
	1'-oxoapiol	2592 \pm 755.7	2.17	0.85		
	1'-sulfoxyapiol				$45 \times 10^{-7} \pm 4 \times 10^{-7}$	0.039

^a Mean values of three independent measurements \pm SD^b Scaled V_{max} were converted from *in vitro* V_{max} -based on microsomal protein yield of 35 mg/g liver in rat and 32 mg/g liver in human and S9 protein yield of 143 mg/g liver.^c Catalytic efficiency (scaled $V_{max}(\text{app})/K_m$).^d First order rate constant for product formation.^e Scaled k were converted from k based on microsomal protein yield of 35 mg/g liver and S9 protein yield of 143 mg/g liver.

Evaluation of apiol PBK model performance

Since there are no available quantitative experimental data on the excretion of the apiol metabolites formed after metabolism in both rat and human exposed to apiol, the performance of the newly developed PBK models for apiol could not be evaluated against *in vivo* data. Therefore the evaluation of the PBK models for apiol was based on the evaluation of the similar PBK models for safrole and the other alkenylbenzenes, for which more *in vivo* data allowing evaluation of the models were available. The performance of the rat PBK model developed for safrole was evaluated by Martati et al. (Martati et al., 2011b) where the predicted and observed levels of a variety of safrole metabolites in plasma or the urine of rats were compared. The predicted and observed safrole metabolite levels were found to be similar (Martati et al., 2011). Besides that, a comparison could be made between the predictions of the developed human PBK model for safrole (Martati et al., 2012) and the known *in vivo* data for human plasma concentrations or the urinary excretion of selected safrole metabolites. Furthermore a comparison of the predictions of the developed PBK models for other alkenylbenzenes (estragole and methyleugenol) (Al-Subeihi et al., 2012, Al-Subeihi et al., 2011, Punt et al., 2008, Punt et al., 2009) and the reported plasma and urine levels of their metabolites could be done to support the validity of developed PBK models for the alkenylbenzenes, thus indirectly supporting the validity of the apiol models. Based on the above considerations showing the validity of developed PBK models for all other alkenylbenzenes, it was concluded that it is a reasonable assumption that these models also adequately describe the metabolite levels formed *in vivo* in both rat and human after ingestion of different oral doses of apiol.

Apiol PBK model predictions for rat and human

Figure 6A shows the PBK model-based predictions for the dose-dependent formation of the different microsomal metabolites of apiol in rat liver. 4-Allyl-3,6-dimethoxybenzene-1,2-diol appears to be the major metabolite formed amounting to 91.5-85 % of the dose with the percentage somewhat decreasing with increasing dose. The percentage of the dose converted to 1'-hydroxyapiol was predicted to increase in a dose-dependent manner from 4 % to about 7 % of the dose at low and high dose levels respectively. Concurrent with the increase in the percentage of the dose that undergoes 1'-hydroxylation of the alkene side chain, a dose-dependent increase from about 4 % to 8 % of the dose converted by epoxidation was observed. Figure 6B shows the PBK model-based predictions for the dose-dependent increase in the formation of the metabolites of 1'-hydroxyapiol in rat liver. The percentage of the dose of apiol converted to 1'-sulfoxyapiol varies with the dose amounting to 0.0032 % at a low dose of 0.05 mg/kg bw to 0.0050 % of the dose at a relatively high dose of 300 mg/kg bw, while these percentages amount to 0.017 % at low dose and 0.026 % at high dose for 1'-oxoapiol. Glucuronidation appears to be the major metabolic route for 1'-hydroxyapiol at all dose levels as 1'-hydroxyapiol glucuronide amounts to about 4.38 % of the dose at low dose levels and to 6.80 % of the dose at high dose levels.

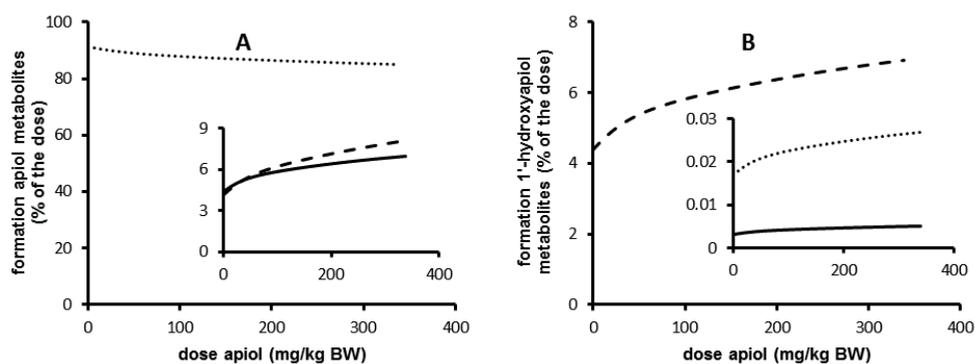


Figure 6. PBK-predicted dose-dependent changes in overall formation of (A) microsomal metabolites of apiol in rat liver and (B) metabolites of 1'-hydroxyapiol in rat liver. The lines correspond to (A) 4-allyl-3,6-dimethoxybenzene-1,2-diol (••••), 1'-hydroxyapiol (—), and 2',3'-dihydroxyapiol (— —), and (B) 1'-hydroxyapiol glucuronide (— —), 1'-sulfoxyapiol (—), and 1'-oxoapiol (••••).

Figure 7A shows the PBK model-based predictions for the dose-dependent formation of the different microsomal metabolites of apiol in human liver. In human liver 4-allyl-3,6-dimethoxybenzene-1,2-diol was predicted to be the major microsomal metabolite amounting to about 82.6-83.5% of the dose. In human liver the percentage of the dose converted to 1'-hydroxyapiol was only slightly decreased with the dose amounting to 6.7-6.3 % of the dose. The decrease in the formation of 1'-hydroxyapiol with increasing dose, is predicted to be accompanied by a decrease in the formation of apiol-2',3'-oxide. Figure 7B shows the dose-dependent decrease in the relative formation of 1'-hydroxyapiol glucuronide and 1'-oxoapiol and the dose-dependent decrease of the relative formation of 1'-sulfoxyapiol in human liver. There were only limited changes in the relative percentage of the different metabolites with the dose. 1'-Hydroxyapiol glucuronide appears to be the major 1'-hydroxyapiol metabolite amounting to about 5.9-5.5% of the dose, while 1'-oxoapiol amounts to about 0.75% of the dose and 1'-sulfoxyapiol to 0.035% of the dose.

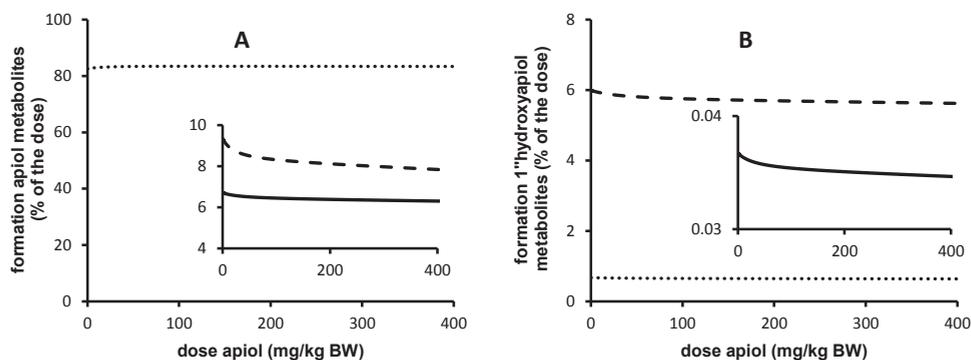


Figure 7. PBK-predicted dose-dependent changes in overall formation of (A) microsomal metabolites of apiol in human liver and (B) metabolites of 1'-hydroxyapiol in human liver. The lines correspond to (A) 4-allyl-3,6-dimethoxybenzene-1,2diol (••••), 1'-hydroxyapiol (—), and 2',3'-dihydroxyapiol (— —), and (B) 1'-hydroxyapiol glucuronide (— —), 1'-sulfoxyapiol (—), and 1'-oxoapiol (••••).

Figure 8 shows a comparison of the relative extent of bioactivation of apiol by rat and human liver. There are species differences in the formation of 1'-hydroxyapiol (Figure 8A) and 1'-sulfoxyapiol (Figure 8B) (expressed as nmol/g liver). In rat liver the formation of 1'-hydroxyapiol at low dose was almost 2 times lower than in human liver and gradually increased with increasing the dose to reach almost the same relative level as in human at high dose. Human liver was predicted to be more efficient in formation of 1'-sulfoxyapiol being 15-fold higher at low dose and 8-fold higher at high dose than 1'-sulfoxyapiol formation by rat liver (Figure 8B). This higher relative formation of 1'-sulfoxyapiol in human than rat liver in spite of the almost equal rate of formation of its metabolic precursor 1'-hydroxyapiol in rat and human liver is due to the fact that the glucuronidation of 1'-hydroxyapiol in rat liver is around 10 times more efficient than that in human liver providing increased opportunity for the competing sulfonation. The difference in overall formation of the 1'-sulfoxyapiol formation between rat and human is not due to a marked difference in the kinetics for

sulfonation of the 1'-hydroxyapiol since this difference appeared limited with an only 1.35-fold higher first order rate constant for sulfonation in rat than human liver (see Table 3).

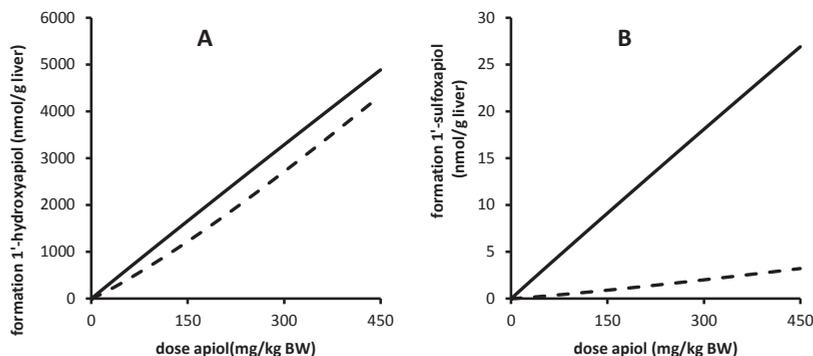


Figure 8. PBK-predicted dose-dependent formation of (A) 1'-hydroxyapiol and (B) 1'-sulfoxyapiol in rat and human liver.

Sensitivity analysis

In order to know the model parameters that have the highest impact on the formation of 1'-hydroxyapiol and 1'-sulfoxyapiol in rat and human liver, a sensitivity analysis was performed. The analysis was done at an apiol dose of 0.05 mg/kg body wt per day to calculate the normalized sensitivity coefficients for all model parameters. Normalized sensitivity coefficient values higher than 0.1, affecting the formation of 1'-hydroxyapiol and 1'-sulfoxyapiol in rat and human liver, are shown in figures 9A and 9B. Figure 9 shows that in both rat and human the parameters that influence the formation of both metabolites to the highest extent are the same. 1'-Hydroxyapiol formation is mainly influenced by the kinetic constants for formation of 1'-hydroxyapiol ($V_{\max,HA}$, $k_{m,HA}$) and the kinetic constants for formation of 4-allyl-3,6-dimethoxybenzene-1,2-diol ($V_{\max,ADD}$, $k_{m,ADD}$). Besides the above

parameters, 1'-sulfoxyapiol formation was influenced by kinetic constants for formation of 1'-hydroxyapiol glucuronide ($V_{\max, \text{HAG}}$, $k_{\text{m, HAG}}$) and of 1'-sulfoxyapiol (k_{HAS}).

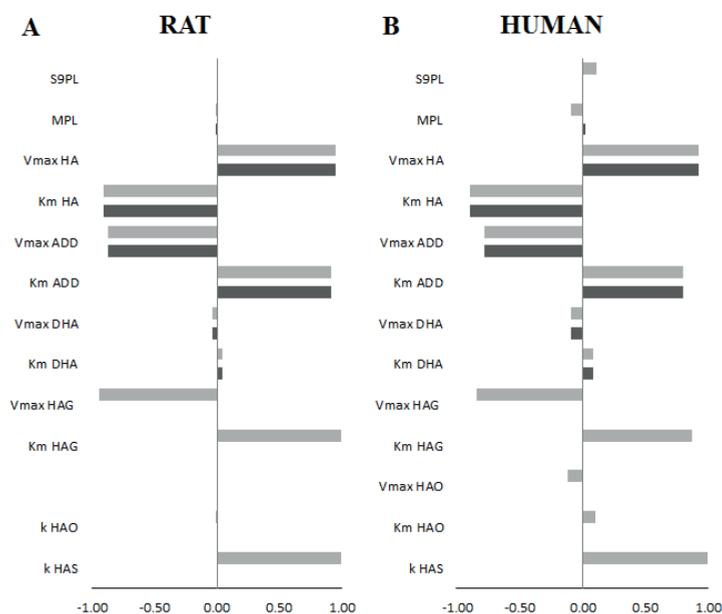


Figure 9. Normalized sensitivity coefficients for the formation of 1'-hydroxy apiol (black) and 1'-sulfoxyapiol (grey) in the liver of rat and human. S9PL = S9 protein yield of the liver, MPL = microsomal protein yield of the liver, Vmax the maximum velocity and Km the Michaelis-Menten constant for the formation of the different metabolites, k = the kinetic rate constant for a first order reaction, ADD = 4-allyl-3,6-dimethoxybenzene-1,2-diol, DHA = 2',3'-di hydroxyapiol, HA = 1'-hydroxyapiol, HAG = 1'-hydroxyapiol glucuronide, HAO = 1'-oxoapiol, HES = 1'-sulfoxyapiol

Comparison of the PBK model-based prediction of bioactivation of apiol by rat and human to the PBK model predictions for bioactivation of the structurally related compound safrole.

The PBK model-based predictions for bioactivation of apiol in rat and human, were compared to the PBK model-based predictions for bioactivation of safrole to facilitate a read-across-based risk assessment. Figure 10 shows the dose-dependent formation of the 1'-hydroxy and

1'-sulfoxy metabolites in rat liver as predicted by the respective PBK models from dose levels representing estimated human daily intakes up to dose levels causing liver tumors in rodent bioassays with safrole. In rat liver the PBK model-based predicted formation of the proximate carcinogenic 1'-hydroxy metabolite was found to be 2.8-fold lower for apiol than for safrole at low dose and 2.0-fold lower at the highest dose (Figure 10A). Figure 10B shows the predicted model outcomes for the formation of the ultimate carcinogenic 1'-sulfoxy metabolites of apiol and safrole. In rat liver the formation of the 1'-sulfoxy metabolite is predicted to be about 3-fold lower for apiol than for safrole. For both alkenylbenzenes the formation of their 1'-sulfoxy metabolite increases linearly with increase of the dose.

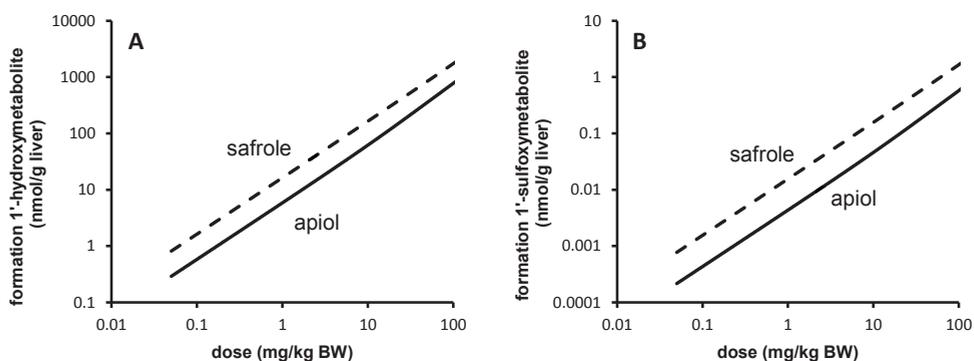


Figure 10. PBK-predicted dose-dependent formation (nmol/g liver) of (A) 1'-hydroxymetabolites and (B) 1'-sulfoxy metabolites of apiol (—) and safrole (---) in male rat liver.

Figure 11 shows the predicted dose-dependent formation of the 1'-hydroxymetabolites and 1'-sulfoxy metabolites of apiol and safrole in human liver. The predicted formation of the 1'-hydroxy metabolite is predicted to be about 8-fold lower for apiol than for safrole at low dose and about 6-fold lower at high dose levels. In human liver the formation of the 1'-sulfoxy metabolites of apiol is predicted to be 2 times lower for apiol than for safrole.

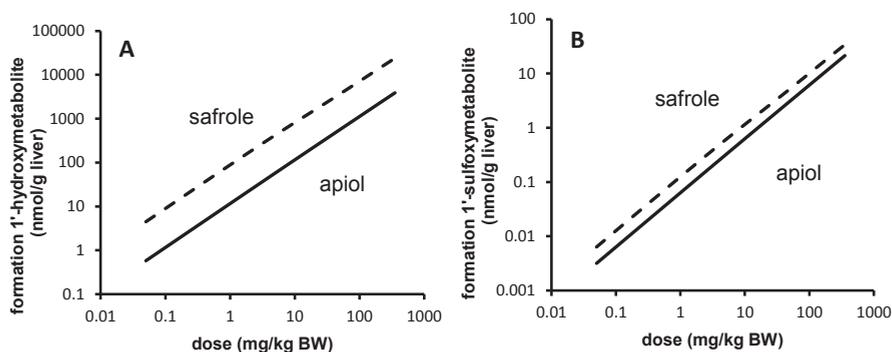


Figure 11. PBK-predicted dose-dependent formation (nmol/g liver) of (A) 1'-hydroxymetabolites and (B) 1'-sulfoxymetabolites of apiol (—) and safrole (---) in human liver.

Implications for risk assessment

In order to evaluate the possible human health hazards due to daily consumption of apiol, the margin of exposure (MOE) approach can be used. The MOE is the preferred approach to assess the safety of a compound that is carcinogenic and genotoxic (EFSA, 2005).

Carcinogenicity data for apiol from which a BMDL₁₀ can be derived are absent, hampering the application of the MOE approach in the risk assessment of apiol. On the other hand, the results of the PBK model predictions presented above indicate that at dose levels in the range at which safrole exposed rodents develop tumors (10-100 mg/kg body wt per day) (Long et al., 1963), the formation of 1'-sulfoxymetabolites of apiol in rat liver is about 3-fold lower than that of safrole. Based on this result it can be assumed that the PBK modelling supports use of the BMDL₁₀ for tumor formation by safrole for risk assessment of the related compound apiol making a correction for the expected 3-fold lower formation of the ultimate carcinogenic 1'-sulfoxymetabolite assuming the same mode of action for these two analogues. Depending on the differences of formation of ultimate carcinogen of apiol and safrole in rat liver at dose

levels that induce liver tumor formation in rodents exposed to safrole, the BMDL₁₀ of safrole which amounts to 1.9-5.1 mg/kg body wt per day (van den Berg et al., 2011) was multiplied by factor of 3 resulting in a BMDL₁₀ of 5.7-15.3 mg/kg body wt per day. The estimated mean daily intake of apiol from spice and spice oil in the USA was reported to amount to 4×10^{-5} mg/kg body wt per day (WHO, 2009). Using a BMDL₁₀ of 5.7-15.3 mg/kg body wt per day and an estimated daily intake of apiol of 4×10^{-5} mg/kg body wt per day the MOE for apiol would amount to 140000 -385000. This indicates that there would be a low concern and a low priority for risk management of the current levels of exposure to apiol. For comparison the MOE values of safrole can be given, obtained at the estimated daily intake of safrole from spice and spice oil in USA that amounts to 1×10^{-3} mg/kg body wt per day (WHO, 2009), and using a BMDL₁₀ of safrole of 1.9-5.1 mg/kg body wt per day. These data result in an MOE for safrole of 1900-5100 indicating a priority for risk management that is higher than for apiol.

DISCUSSION

The present paper describes the definition of mode of action-based PBK models for detoxification and bioactivation of the alkenylbenzene apiol in rat and human and the use of predictions made by these models to facilitate a read-across-based risk assessment to the structurally related alkenylbenzene safrole (Martati et al., 2011, Martati et al., 2012), for which *in vivo* carcinogenicity data are available. Previously, using a similar approach and read-across to estragole and methyleugenol, the risks of exposure to elemicin for which also no *in vivo* rodent tumor data were available could be evaluated (Van den Berg et al., 2012). The approach enables a preliminary risk assessment of apiol for which *in vivo* toxicity data are limited without the need for additional animal studies. The physiological parameters for rat and human and the biochemical and physiochemical parameters for apiol were defined to

develop the apiol PBK models. The newly developed PBK models for apiol provide insight into the species-differences in the relative extent of bioactivation and detoxification, and could also be used to compare the level of metabolic activation of apiol to that of safrole in male rat and human liver (Martati et al., 2011b, Martati et al., 2012b). Comparing the apiol and safrole model-based predictions showed that the formation of the proximate and ultimate carcinogenic metabolites of the two alkenylbenzenes in rat is predicted to be comparable within an order of magnitude. The PBK models predict the formation of the ultimate carcinogenic 1'-sulfoxymetabolite at a dose level of 100 mg/kg body wt, at which safrole is causing liver tumors in rodent animal (Long et al., 1963, WHO, 2009), to be 3-fold lower for apiol than for safrole in rat liver. In line with these differences, Randerath et al. (Randerath et al., 1984), using ³²P-postlabeling reported that the number of DNA adducts formed by apiol in adult mouse liver was lower than for safrole. In a parallel study, Phillips et al. (Phillips et al., 1984) reported that in preweaning male mice the level of DNA adducts formed after neonatal apiol exposure was about 12-fold lower than after neonatal safrole exposure (Phillips et al., 1984). In addition, the ability of apiol to form DNA adducts in cultured human (HepG2) cells was reported to be 3-5 times lower than that of safrole (Zhou et al., 2007).

It is important to note that the PBK model-based predictions quantify the amount of the ultimate carcinogenic metabolite formed and not the ability of these compounds to bind to DNA or other macromolecules. One could argue that this reactivity may differ between the different alkenylbenzene compounds and that an increasing number of methoxy groups may reduce the electrophilicity of the 1'-sulfoxymetabolite or its carbocation decreasing its ability to bind to DNA (Miller et al., 1983, Randerath et al., 1984). However, given that the PBK model-based predicted differences are in line with the currently available data from *in vivo* and cellular *in vitro* studies on DNA adduct formation by apiol and safrole it is concluded that

these possible differences in electrophilicity of the two 1'-sulfoxymetabolites does not affect the predictions to an extent that it would modify the conclusions of the current MOE based preliminary risk assessment.

Based on the rat PBK model predictions for formation of the related 1'-sulfoxymetabolite, being 3-fold lower for apiol than for safrole at dose levels that are known to induce tumors in safrole-exposed rodent models, it was concluded that the BMDL₁₀ that would be expected in a rodent animal bioassay for apiol could be 3-fold higher than that of safrole. Based on these considerations a preliminary risk assessment was performed for apiol using the MOE approach and an estimated BMDL₁₀ for apiol, of 3 times the BMDL₁₀ for safrole (1.9-5.1 mg/kg body wt per day) (Suzanne J. P. L. van den Berg et al., 2011) and the MOE for apiol was calculated to amount to 140000 -385000. It is of interest to note that the PBK model of the present study predicted that the formation of the ultimate carcinogenic 1'-sulfoxymetabolite of apiol in human liver would be about 10 times higher than its formation in rat liver. This difference is included in the MOE of 10000 which was defined to include a factor of 10 for interspecies differences (EFSA, 2005). Given that the MOE values actually obtained for apiole are 14-38-fold higher than 10000 they could accommodate an even larger species difference than the 10-fold difference now included in the MOE of 10000 without leading to a different conclusion. Furthermore, comparison of the MOE values obtained in the present study for apiol and safrole, indicate a priority for risk management of safrole that is higher than for apiol, due to its 3 times lower BMDL₁₀ and its 25-fold higher estimated daily intake.

In conclusion, the present study reports the development of apiol PBK models for rat and human providing insight into the occurrence of species differences in the metabolic activation of this food-borne compound that is genotoxic and carcinogenic. In addition the PBK model predictions were applied to support read-across from safrole for which tumor data are

available facilitating a preliminary risk assessment for apiol for which limited *in vivo* data are available. This example illustrates how PBK modelling can be used to contribute to risk assessment eliminating the need for animal bioassays thereby contributing to the 3Rs of animal testing in human safety assessments.

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Chapter 4

Determination and risk assessment of naturally occurring genotoxic and carcinogenic alkenylbenzenes in basil-containing sauce of pesto

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ABSTRACT

A risk assessment of basil-based pesto sauces containing methyleugenol and related alkenylbenzenes was performed based on their levels detected in a series of pesto sauces available on the Dutch market. The estimated daily intake (EDI) values of alkenylbenzenes as a result of consumption of the different pesto sauces amounted to 1.2–44.3 $\mu\text{g}/\text{kg}$ bw for individual alkenylbenzenes, 14.3–43.5 $\mu\text{g}/\text{kg}$ bw when adding up the alkenylbenzene levels assuming equal potency, and 17.3–62.9 $\mu\text{g}/\text{kg}$ bw when expressed in methyleugenol equivalents using alkenylbenzenes defined toxic equivalency factors (TEF). The margin of exposure approach (MOE), used to evaluate the potential risks, resulted in MOE values that were generally lower than 10000 indicating a priority for risk management when assuming daily consumption. The levels of methyleugenol detected in the pesto sauces would allow consumption of 1.1–29.8, 7.5–208, 15.1–416.5, and 32.4–892.5 g of pesto sauce on a daily basis, once a week, once every two weeks, and once a month, respectively, to achieve MOE values above the 10000 limit indicating low priority for risk management. It is concluded that consumption of pesto sauces would only be of concern if consumed on a daily basis over longer periods of time.

Abbreviations

EDI, estimated daily intake; BMDL₁₀, lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence; EFSA, European Food Safety Authority; ESCO, scientific cooperation projects between EFSA and its counterparts in the Member States; MOE, margin of exposure; UPLC, Ultra Performance Liquid Chromatography; TEF, toxic equivalency factor; TEQ, toxic equivalency

Keywords

Margin Of Exposure (MOE); Basil-based pesto; combined risk assessment; Toxic Equivalency Factors (TEFs)

INTRODUCTION

The genus *ocimum*, collectively called basil, belongs to the *Lamiaceae* family (the mint family), a family that contains 236 genera (Harley et al. 2004) and 7534 species (Kew). The plants are aromatic and cultivated for culinary purposes. Basil adds a distinctive flavor to many foods, and is consumed fresh, dried, as essential oil, or processed. In its processed form, basil, especially *Ocimum basilicum* cv. Genovese gigante cultivar, is employed in the production of pesto, a typical Italian sauce. The worldwide consumption of pesto has increased due to recent interest in Mediterranean cuisine, which is considered to be healthy and valuable. The complex matrix of pesto (e.g. cheese, extra-virgin olive oil, pine nuts and/or walnuts, and garlic) significantly affects its taste and flavor (Salvadeo et al. 2007). Previously, the aromatic composition of basil was determined from different areas of north western Italy and showed methyleugenol (1,2-dimethoxy-4-prop-2-en-1-yl-benzene) and eugenol as main components (Miele et al. 2001), while estragole was shown to be present in pesto sauces made of basil cultivars different from the Genovese gigante cultivar (Marotti et al. 1996; Siano et al. 2003). In 1999, the Committee of Experts on Flavoring Substances of the Council of Europe concluded that methyleugenol is a naturally-occurring genotoxic carcinogenic compound with a DNA-binding potency similar to that of safrole (EC 2001). In 2001, the Scientific Committee on Food (SCF) of the European Union concluded that methyleugenol, and its structural analogues estragole and safrole (Figure 1) are genotoxic and carcinogenic and that reductions in exposure and restrictions in use levels are indicated (SCF 2001a; SCF 2001b; SCF 2002). Rodent studies performed at high dose levels provided sufficient evidence of the hepatocarcinogenicity of methyleugenol, estragole and safrole, displaying similar characteristics regarding mode of action and tumor formation (Phillips et al. 1981; Miller et al. 1983; Randerath et al. 1984; NTP 2000; Zhou et al. 2007; Kobets et al. 2016). EC Regulation 1334/2008, which became effective in January 2011, prohibits the

addition of methyleugenol, estragole and safrole to foods and restricts their concentration in compound foods of dairy products, meat preparations and meat products (including poultry and game), fish preparations and fish products, ready to eat savouries, non-alcoholic beverages, processed fruit and vegetables, and soups and sauces including pesto. For soups and sauces including pesto that have been prepared with flavorings or food ingredients with flavoring properties maximum allowed limits of 60 mg/kg for methyleugenol and 25 mg/kg for safrole have been defined while a limit for estragole has not been presented (EC 2008). However, if the only food ingredients with flavoring properties that have been added are only the fresh, dried or frozen herbs, the maximum limit for methyleugenol does not apply. This implies that pesto sauce made with basil is permitted in food preparations, regardless of its genotoxic carcinogenic compounds content.

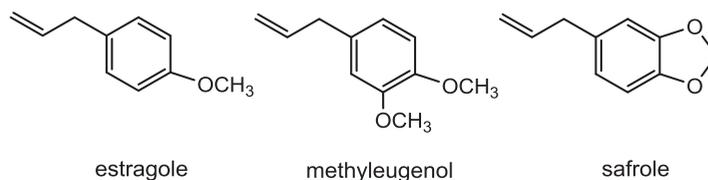


Figure 1. Structural formula of methyleugenol and its structural analogues.

A major issue in the safety assessment of alkenylbenzene-containing food items is how to provide guidance on the potential risks for human health resulting from the exposure to low levels of these genotoxic and carcinogenic compounds (EFSA 2005). The European Food Safety Authority (EFSA) recommended the Margin of Exposure (MOE) approach (EFSA 2005) to evaluate the priority for risk management actions for compounds that are both genotoxic and carcinogenic and present in a botanical or botanical preparation of interest (EFSA 2009a; EFSA 2009b). The MOE compares the exposure levels causing

malignant tumors in experimental animals with dietary intake estimates in humans (EDI), taking into account differences in consumption patterns. The Scientific Committee of the European Food Safety Authority recommends the use of the benchmark dose (the BMDL₁₀; the lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence) to obtain the MOE (EFSA 2005). The benchmark dose is a standardized reference point derived from the animal data by mathematical modelling within the observed range of experimental data (EFSA 2009b). Pesto eaters could be exposed to some of the highest levels of methyleugenol, because fresh pesto is prepared from a large quantity of fresh basil, reflected by basil amounts in pesto sauce products on the market that amounted on average to 36% (an average from different products in the market). It has been estimated that the theoretical dietary exposure to methyleugenol could reach ~250 µg/kg bw per meal for adults and 500 µg/kg bw per meal for children, based on the assumptions that the percentage of methyleugenol in the essential oil is generally >40 %, the amount of essential oil in *O. basilicum* cv. Genovese gigante corresponds 0.5 % and that one portion of pesto contains ~10 g of basil (Miele et al. 2001). The French food control administration analysed methyleugenol in a limited number of well-defined food products and reported that the methyleugenol concentration for one sample of pesto sauce was 6.02 µg/g and for one sample of pasta with pesto was 1.53 µg/g (Smith et al. 2010). Ávila et al. reported methyleugenol concentrations in a pesto sample amounting to 48 µg/g (Avila et al. 2009), and Siano et al. reported methyleugenol concentrations of 0.01–0.52 µg/g and estragole concentrations that amounted to 0.05–19.30 µg/g (Siano et al. 2003). It appears that the current values reported on levels of the alkenylbenzenes in sauces of pesto are limited with values that vary orders of magnitude. The aim of the present study was to make a more detailed analysis of the presence of methyleugenol and possible other alkenylbenzenes in basil-containing sauce of pesto and perform an associated risk assessment based on the MOE approach, taking into consideration

the possible combined exposure to different alkenylbenzenes. Given that the alkenylbenzenes are known to act by a similar mode of action, at a similar target organ causing the same adverse effect, being liver carcinogenicity caused by a genotoxic mode of action, combined exposure can be evaluated by dose addition. This implies that the response to the mixture can be predicted by summing the doses of the components if needed after adjusting for the differences in potencies. Dose addition is considered most appropriate for mixtures with components that affect the same endpoint by the same mode of action (EPA 1986a; EPA 1988; EPA 2000). Dose addition is the underlying assumption of the toxic equivalency factor (TEF) approach (ATSDR 2004).

MATERIALS AND METHODS

Pesto sauce samples and chemicals

Basil containing pesto sauce samples were purchased from local Dutch markets. Table 1 presents an overview of the collected samples and their major characteristics as indicated on the label of each product. Methyleugenol, myristicin, estragole and apiol (purity, >97%), methanol (HPLC gradient) and acetonitrile (LC/MS gradient) were supplied by Sigma Aldrich (Steinheim, Germany). Nano pure water was obtained from a Barnstead Nanopure Type I ultrapure water system. Dimethyl sulfoxide (DMSO) was obtained from Acros organics (Geel, Belgium). Acetonitrile (ACN) (LC/MS grade) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Trifluoroacetic acid TFA was obtained from Merck (Darmstadt, Germany).

Table1. Basil-containing pesto sauce samples used in the present study and their major characteristics. Information provided was derived from the product labels unless stated otherwise.

Sample no.	Description	Ingredients
1	Pesto, Italian basil sauce, 85 g	Cold-pressed olive oil, fresh basil , fresh garlic, pine nuts, sea salt
2	Genovese pesto, biological, totally vegetarian, cheese free, gluten free, 130 g	Extra virgin olive oil 36%, Genovese basil in extra virgin olive oil 27.6%, salt, cashew nuts, walnuts, pine kernels, apple fibre, garlic, acidity
3	Combined with dried tomatoes and a hint of spice routes, biological, 135 g	Sunflower oil, breadcrumbs, cashew, basil , honey, garlic, olive oil, dried tomatoes (22.5%), vinegar, sea salt, natural flavour, pepper, thyme, paprika powder, cumin, chili pepper
4	Traditional terrasa pesto, bio, organic, 180 g	Extra virgin olive oil, fresh basil (35%) , pecorino sheep cheese (6%), pine kernels (5%), garlic (3%), sea salt
5	Genovese pesto, costa ligure, 135 g	Sunflower oil, fresh basil 73% , salt, cashew, pine nuts, parmesan cheese, garlic, wine vinegar, starch, acidity regulators of critic acid, lactic acid
6	Green pesto, 190 g	Sunflower oil, basil 36% , cashew nut, whey powder, cheese, salt, potato, sugar, acid regulator, dried garlic
7	Italian Genovese pesto, 90 g	Sunflower oil, basil 35% , glucose, potato flake, cashew nut, milk, salt, rennet, preservatives
8	Excellent Genovese pesto, 135 g	Extra virgin olive oil, sunflower oil, fresh basil 69% , pine nut 13%, parmesan.
9	Tartufo pesto, costa ligure, 135 g	Sunflower oil, basil 42% , salt, cashew, pine nuts, parmesan cheese, garlic, wine vinegar, starch, acidity regulators of critic acid, lactic acid, 1% truffle
10	Limon pesto, 135 g	Oiled sunflower, fresh basil 59% , salt, cashew, pine nuts, parmesan cheese, garlic, wine vinegar, starch, acidity regulators of critic acid, lactic acid, 1.5 citron
11	Genovese pesto, 185 g	Sunflower oil (34%), basil 29% , potatoes, corn syrup, cashew nuts, grana padano cheese (4.5%), pecorino cheese (4.5%), salt, extra virgin olive oil, pine nuts (1%), natural flavours, garlic, concentrated lemon juice

Table1. (Continued) Basil-containing pesto sauce samples used in the present study and their major characteristics. Information provided was derived from the product labels unless stated otherwise.

Sample no.	Description	Ingredients
12	Italian arugula pesto, 190 g	Sunflower oil, arugula 30%, basil 8% , pecorino romano cheese, sea salt, garlic, vegetable fibres, pine nuts, extra virgin olive oil, acidity regulator: lactic acid
13	Biological green pesto, 135g	Sunflower oil, fresh basil 79%, salt 1%, pine nuts, garlic, white wine vinegar, acid regulator (lactic acid, citric acid)
14	The real Italian seasoning, green pesto, 190 g	Basil 42%, sunflower oil, cashew nut 6.5%, water, parmigiano reggiano cheese 2.5%, salt, garlic, yogurt powder, olive oil 0.5%, pine 0.5%, acidity regulator: lactic acid, natural basil flavor, natural white pepper flavor, whey protein, antioxidant: E300
15	Green pesto, classic basil pesto, garlic and parmesan cheese, 125 g	Basil 42%, sunflower oil 32.2%, cashew nuts, parmesan 4% (milk, salt, rennet), salt, pecorino cheese 2%, garlic 1.5%, glucose syrup, milk protein, acidity regulator: lactic acid, extra virgin olive oil, pine 0.5%, natural flavor
16	Green pesto, with more basil, 185 g	Basil 46% , sunflower oil, Italian cheese 7% (grana padano, pecorino romano), cashews, salt, extra virgin olive oil with virgin olive oil 2%, glucose syrup, sugar, potato flakes, flavor, garlic 0.5%, pine nut 0.5%, lactic acid
17	Green vegan pesto with extra virgin oil, bio, organic, 180 g	Extra virgin oil, basil 30% , walnuts, cashew nuts, pine kernels, sea salt
18	Italian green pesto, 90g	Basil 39% , 27% sunflower oil, water, lactose, 4% cashews, 4% parmigiano reggiano, 2.8% olive oil, 2% pine nuts, salt, spices extract, 1% pecorino cheese, garlic, acid: E270, natural flavoring
19	Fresh green pesto, in-house made (supermarket), 100 g	Sunflower oil, basil 23.7% , 9.5% cheese powder, walnuts, parsley, salt, vinegar, garlic 2%, 1.9% pine nuts, sugar water, white pepper, food acids: lactic acid and citric acid, thickening agent, E415 and E415, preservative E202
20	Artisan and full of flavor green pesto, 100 g	Sunflower oil, basil 30% , 8.5% grana padano cheese, cashews, olive oil, salt, pecorino cheese, fructose, pine, bamboo fiber, garlic, antioxidant E300
21	Creamy green pesto with basil, cheese and pine nuts, 125 g	Vegetable oil (27% olive, rapeseed), basil 22% , 11% cheese powder, 7% pine nuts, parsley, 2.5% garlic puree, white wine vinegar, salt, sugar, vinegar, natural flavoring, preservative (E200, E210), black pepper, thickening agent (guar gum, xanthan gum)

Table1. (Continued) Basil-containing pesto sauce samples used in the present study and their major characteristics. Information provided was derived from the product labels unless stated otherwise.

Sample no.	Description	Ingredients
22	Creamy red pesto with tomato, cheese and pine nuts, 125 g	Vegetable oil (13% olive, rapeseed), 16 tomatoes, basil 14% , 12% cheese powder, 8% pine nuts, parsley, 3% garlic puree, white wine vinegar, salt, sugar, vinegar, natural flavoring, preservative (E200, E210), thickener (guar gum, xanthan gum), 1.5% sundried tomatoes, red peppers
23	Pesto with Sicilian pistachio nuts, 130 g	Olive oil, Genovese basil 22% , pistachio nut (18%), cashew nuts, sea salt
24	Pesto with lemon zest, 135g	Olive oil, fresh basil 59% , cashew nuts, salt, cheese mix (2%), limon rinds (1.5%), fructose, pine nuts, wine vinegar, garlic, natural lemon flavoring, acidity regulator citric acid, lactic acid
25	Pesto with basil & truffle, 130g	Olive oil, Genovese basil 22% , cashew nuts, sea salt, pine nuts, white truffle
26	Rosso pesto with sun dried tomato and basil, 130g	Olive oil, sun dried tomato 25%, Genovese basil 25% , cashew nuts, sea salt, pine nuts, acidity regulator citric acid, lactic acid
27	Freshly made pesto, from Italian food products shop	Private recipe
28	Freshly made pesto, from cheese shop	Private recipe
29	Pesto spread, 90g	Sunflower oil, 15% pine nuts, low-fat cream cheese, basil 9% , spinach, white wine vinegar, 2.5 pecorino, garlic, salt, emulsifier (E472), bovine gelatin, preservative (E202, E224)
30	Special basil pesto, 100g	Sunflower oil, pine nut 15%, 12% old cheese, basil 11% , 10% spinach, white wine vinegar, dried garlic, sea salt, preservative (E202, E224)
31	Bio basil pesto, 100g	Basil 32% , sunflower oil, cheese, olive oil 55, 4% pine nuts, vinegar, garlic, lemon juice, sea salt, spices, coconut blossom sugar

Methanol extracts

All basil-containing pesto sauces were extracted using methanol based on the method described by Ávila et al. (Ávila et al. 2009), with minor modifications. In short, 5 g of each sauce sample were sonicated in an ultrasonic bath for 10 min and macerated for 12-hr at 50°C with 80 ml of methanol in a closed glass vessel. Finally, an aliquot of the extract solution was centrifuged at 13000×g for 5 min, and stored at -20°C until Ultra Performance Liquid Chromatography (UPLC) analysis. Samples were extracted and then analyzed on UPLC in triplicate. The accuracy of the developed method was assessed by means of a recovery study and standard deviations of the replicates. For recovery, pure standards of commercially available alkenylbenzenes were added in two different quantities (final concentration of 5 and 10 µM) to 5 g of two pesto sauce samples (sample 16 and 21) which covers the whole range of analysis in a final volume of methanol of 80 ml. Samples were prepared and analyzed as described above to quantify the alkenylbenzene levels enabling determination of the recovery. The extraction capacity linearity was demonstrated to applying a range of weight of sample to final volume of the solution ratio of 1.5 % up to 10 %.

UPLC analysis

For quantification of alkenylbenzenes in the extracts, 3.5 µl of each sample was subjected to UPLC analysis. The UPLC system consisted of a Waters (Waters, Milford, MA) Acquity solvent manager, sample manager, and photodiode array detector. Chromatographic separation was achieved using an ACQUITY UPLC BEH C18 1.7 µm column, 2.1 × 50 mm. The column was thermostated at 35 °C and the sample manager was set at 10 °C. An isocratic gradient was made for the separation using a mixture of acetonitrile (ACN) and ultrapure water containing 0.1 % (v/v) trifluoroacetic acid (TFA). The mobile phase consisted of 40% acetonitrile for 4 min, which was the time needed to separate alkenylbenzenes of interest.

During the whole run, the flow rate was 0.6 ml/min. Under these conditions, the retention times for methyleugenol, estragole, myristicin, and apiol were 1.5, 2.2, 2.4, and 2.8 min, respectively. Identification of the alkenylbenzenes was achieved by comparing the UV spectrum and retention time of the peak to the UV spectrum and retention time of the peaks of commercially available reference compounds. Quantification was done by comparing the area of the alkenylbenzene peak to that of the calibration curve of the reference compounds determined using UPLC with photodiode array detection (UPLC/PDA) at a wavelength 201 nm for methyleugenol and estragole, and a wavelength 210 nm for myristicin and apiol.

Determination of estimated daily intake (EDI) of alkenylbenzenes resulting from the use of pesto sauce

The estimate daily intake (EDI) of alkenylbenzenes resulting from the use of pesto sauce was determined based on the alkenylbenzene content in the pesto sauce as determined in the present study (see results) and a body weight of 70 kg, the default value for adult body weight proposed by EFSA (EFSA 2012b). Daily consumption of pesto sauce was based on the reported use of about 10 g basil for a meal (Miele et al. 2001), and on the basil content of the samples as mentioned on the label (Table 1). If there was no information on the basil content in the pesto sauce on the label the average content of basil in pesto sauce of $36.0 \pm 19.6\%$ ($n = 27$) (Table 1) was used. Firstly, the EDI was calculated for individual compounds for all of the samples. And since several alkenylbenzenes were found in some of the pesto samples, and the different alkenylbenzenes show similarity in structure, target organ, type of adverse effects and mode of action through formation of a DNA reactive 1'-sulfoxymetabolite contributing to formation of liver tumors, a combined exposure assessment and subsequent risk assessment were also performed for those samples. To this end two approaches were used; summing up the EDIs of the alkenylbenzenes assuming equal potency, and a so-called

toxic equivalency (TEQ) approach calculating the estimated daily intake (EDI) values of the alkenylbenzenes using their toxic equivalency factor (TEF) relative to methyleugenol. Methyleugenol was taken as the reference compound (TEF = 1.0), expressing the concentrations of the other alkenylbenzenes in methyleugenol equivalents. TEF values for the different alkenylbenzenes for the TEQ approach need to be defined, which was done by calculating an average of three endpoints. The first one was the slope of the dose-response curve for DNA-adduct formation in female CD-1 mice liver upon exposure to different alkenylbenzenes (Randerath et al. 1984). The second one, was the *in vivo* level of formation of the hepatocarcinogenic 1'-sulfoxymetabolites of the different alkenylbenzenes predicted using human physiologically based kinetic (PBK) models at a dose level of 0.01 mg/kg bw (Punt et al. 2009; Al-Subeihi et al. 2012; Alajlouni et al. 2016a; Al-Malahmeh et al. 2017). And finally, the BMDL₁₀ values derived from *in vivo* tumor data for safrole, estragole, and methyleugenol (Miller et al. 1983), or by read-across from safrole for myristicin and apiol (Alajlouni et al. 2016a; Al-Malahmeh et al. 2017) (Table 2). The TEF values for the different alkenylbenzenes defined by the three approaches relative to methyleugenol were subsequently averaged to define the final TEF values. Using these TEF values the EDI of alkenylbenzenes in the pesto sauces expressed in (µg methyleugenol equivalents/kg bw per day) was calculated using the formula:

$$EDI (\mu\text{g methyleugenol equivalents})/(\text{kg bw day}) = \sum(EDI \times TEF)_i,$$

i; different alkenylbenzenes

Table 2. Data used for estimation of the TEF values for the different alkenylbenzenes as derived from data reported for i) the dose-dependent level of DNA adduct formation in the liver of alkenylbenzene exposed female CD-1 mice (Randerath et al. 1984) ii) the formation of the proximate carcinogenic 1'-sulfoxy metabolites in liver in $\mu\text{mol/kg bw}$ as predicted by available PBK models at a dose level of 0.01 mg/kg bw (Punt et al. 2009; Al-Subeihi et al. 2012; Alajlouni et al. 2016a; Al-Malahmeh et al. 2017), and iii) BMDL₁₀ values reported in the literature (van den Berg et al. 2011; Alajlouni et al. 2016a; Al-Malahmeh et al. 2017). The TEF values were calculated relative to methyleugenol as the reference compound, expressing the concentrations of the other alkenylbenzenes in methyleugenol equivalents.

Compound	Formation of DNA adduct in 10^7 nucleotides versus dose in mg/kg bw		Formation of the proximate carcinogenic 1'-sulfoxy metabolite		<i>In vivo</i> tumor data		Average TEF value \pm SD
	slope of the curve	TEF	formation level ($\mu\text{mol/kg bw}$) at 0.01 mg/kg bw dose	TEF	BMDL ₁₀ (mg/kg bw per day)	TEF	
Safrole	1.68	1.05	3.28E-05	1.08	1.9	8.05	3.39 \pm 4.04
myristicin	0.39	0.24	2.44E-05	0.8	1.9	8.05	3.03 \pm 4.36
apiol	0.11	0.07	1.62E-05	0.53	5.7	2.68	1.09 \pm 1.40
estragole	2.48	1.55	14.1E-05	4.62	3.3	4.64	3.60 \pm 1.78
methyleugenol	1.60	1.00	3.05E-05	1.00	15.3	1.00	1.00

Calculation of the margin of exposure

Risk assessment of consumption of the pesto sauce samples was performed using the Margin of Exposure (MOE) approach (EFSA 2005). For the individual alkenylbenzene approach, the BMDL₁₀ values of each compound were used (van den Berg et al. 2011; Alajlouni et al. 2016a; Al-Malahmeh et al. 2017) and then compared with the EDIs of the alkenylbenzenes resulting from the use of pesto sauce. For the equal potency assumption approach, the BMDL₁₀ value of the predominating alkenylbenzene in the samples was used and compared to the sum of the EDIs. And finally, for the TEQ approach, the BMDL₁₀ of the reference compound methyleugenol was compared to the EDI expressed in methyleugenol equivalents.

RESULTS

Determination of methyleugenol content in the basil-containing pesto sauce samples, EDI, and resulting MOEs

Table 3 presents the levels of methyleugenol detected in the various pesto sauces. The results obtained revealed that methyleugenol was detected in all the samples analyzed. The level of methyleugenol in the different samples varied about 28-fold ranging between 3.6 and 99.3 $\mu\text{g/g}$ pesto sauce. Estimated daily intakes (EDIs) of methyleugenol from basil containing pesto sauce were calculated using the methyleugenol content in the samples, a daily consumption of pesto calculated based on the reported use of about 10 g basil for a meal (Miele et al. 2001), and the basil content of the samples as mentioned on the label (Table 1) or the assumption that the basil content in pesto sauce was on average $36.0 \pm 19.6\%$ ($n = 27$) (Table 1) in case this % of basil in the pesto sauce was not mentioned on the label, and an adult body weight of 70 kg. The EDI values thus obtained for methyleugenol vary in the range of 3.2–44.3 $\mu\text{g/kg}$ bw per day. The MOE values were calculated using the BMDL_{10} value reported in the literature for methyleugenol of 15.3 mg/kg bw per day (van den Berg et al. 2011) (Table 3). The MOE values obtained vary from 345 to 4781 and were all lower than 10000, the value suggested by EFSA to judge if there is a priority for risk management (EFSA 2005).

Table 3. Level of methyleugenol detected in the pesto sauce samples, EDI for methyleugenol upon consumption of these pesto samples, and the resulting MOE.

Sample no.	Methyleugenol concentration in the pesto sample Average \pm SD ($\mu\text{g/g}$)	EDI ($\mu\text{g/kg bw per day}$)	MOE
1	17.9 \pm 2.4	7.1	2154
2	11.4 \pm 1.5	5.9	2593
3	11.1 \pm 1.5	4.4	3474
4	35.6 \pm 4.8	14.5	1053
5	32.3 \pm 4.3	6.3	2421
6	41.2 \pm 5.5	16.4	936
7	29.6 \pm 4	12.1	1266
8	27.6 \pm 3.7	5.0	3066
9	25.3 \pm 3.4	8.6	1778
10	38.1 \pm 5.1	9.2	1659
11	20.5 \pm 2.7	10.1	1515
12	3.8 \pm 0.5	6.8	2255
13	34.1 \pm 4.6	6.2	2481
14	60.5 \pm 8.1	20.6	744
15	21.8 \pm 2.9	7.4	2063
16	39.0 \pm 5.2	12.1	1263
17	12.4 \pm 1.7	5.9	2591
18	35.8 \pm 4.8	13.1	1167
19	56.4 \pm 7.5	34.0	450
20	15.2 \pm 2.0	7.2	2114
21	22.9 \pm 3.1	6.4	2404
22	15.1 \pm 2.0	14.9	1029
23	8.4 \pm 1.1	15.4	993
24	33.0 \pm 4.4	5.5	2805
25	9.8 \pm 1.3	8.0	1915
26	5.6 \pm 0.7	3.2	4781
27	14.3 \pm 1.9	5.7	2696
28	13.4 \pm 1.8	5.3	2877

Table 3. (Continued) level of methyleugenol detected in the pesto sauce samples, EDI for methyleugenol upon consumption of these pesto samples, and the resulting MOE

Sample no.	Methyleugenol concentration in the pesto sample Average \pm SD ($\mu\text{g/g}$)	EDI ($\mu\text{g/kg bw per day}$)	MOE
29	3.6 \pm 0.5	5.7	2678
30	3.8 \pm 0.5	4.9	3100
31	99.3 \pm 13.3	44.3	345

Determination of other alkenylbenzenes in the pesto sauce samples, EDI for the combined exposure, and resulting MOEs

In four samples (16, 18, 19, and 21) other alkenylbenzenes were detected in addition to methyleugenol, including estragole, myristicin, and/or apiol (Table 4). The levels of methyleugenol, detected in these samples, were already presented in Table 3. Estragole that is known to be present in some basil cultivars in addition to methyleugenol, was detected in two samples (16,18), with one of these samples indeed containing another cultivar than the Genovese cultivar as mentioned on the label (Table 1). The estragole level varied 10-fold between the two samples in which it was detected. Myristicin and apiol were detected in two samples of green pesto (Table 4), which may be due to the fact that these samples contain other herbs in addition to basil (e.g. parsley) (Table 1). For samples 16, 18, 19 and 21 the EDI for the individual alkenylbenzenes varied between 6.4–34.0 $\mu\text{g/kg bw per day}$ for methyleugenol, 1.2–10.6 $\mu\text{g/kg bw per day}$ for estragole, 8.6–9.6 $\mu\text{g/kg bw per day}$ for myristicin, and 2.2 $\mu\text{g/kg bw per day}$ for apiol the latter being detected in only one sample (Table 4).

Table 4. Level of alkenylbenzenes methyleugenol, estragole, myristicin, and apiol in the four pesto sauce samples that contained other alkenylbenzenes in addition to methyleugenol and EDI values for the individual alkenylbenzenes, as well as combined EDI values calculated either by assuming equal potency, or in methyleugenol equivalents using the TEF values presented in Table 2.

Sample no.	Alkenylbenzenes level in the pesto sample average \pm SD ($\mu\text{g/g}$)				EDI ($\mu\text{g/kg bw per day}$)					
	methyleugenol	estragole	myristicin	apiol	individual				combined exposure	
					methyleugenol	estragole	myristicin	apiol	assuming equal potency	based on TEF methyleugenol equivalents
16	39.0 \pm 5.2	34.1 \pm 2.8			12.1	10.6			22.7	50.3
18	35.8 \pm 4.8	3.2 \pm 1.5			13.1	1.2			14.3	17.3
19	56.4 \pm 7.5		15.8 \pm 0.01		34		9.6		43.5	62.9
21	22.9 \pm 3.1		13.2 \pm 1.2	3.4 \pm 0.2	6.4		8.6	2.2	17.2	34.8

Since several alkenylbenzenes were found in some pesto samples, and because these alkenylbenzenes are known to display similarity in structure, target organ, type of adverse effects and mode of action through formation of a DNA reactive 1'-sulfoxymetabolite contributing to formation of liver tumors, a combined exposure and risk assessment was also performed. To this end the combined exposure was estimated in two ways, assuming either equal potency or using a so-called toxic equivalency (TEQ) approach. When assuming equal potency of the different alkenylbenzenes the combined EDI can be calculated by adding up the EDIs of the different alkenylbenzenes. Table 4 presents the combined EDI values obtained for the four pesto sauce samples in which more than one alkenylbenzene were detected. The values obtained varied from 14.3 to 43.5 mg/kg bw per day with methyleugenol being the major alkenylbenzene making up 53.3–91.6% of these values except for sample 22 where methyleugenol makes up 37.2% of the alkenylbenzenes and myristicin appeared to be the major one at 50.0%. In a second approach the combined exposure was calculated in methyleugenol equivalents taking the relative potency of the different alkenylbenzenes into

account. For this TEQ approach the required toxic equivalency factor (TEF) relative to methyleugenol were estimated as summarized in Table 2. TEF values for the different alkenylbenzenes were defined by calculating the average of the relative potency as available for three end points, including (i) the slope of the dose-response curve for DNA-adduct formation in female CD-1 mice liver upon exposure to different alkenylbenzenes (Randerath et al. 1984), (ii) the *in vivo* level of formation of the hepatocarcinogenic 1'-sulfoxymetabolites of the different alkenylbenzenes predicted using human physiologically based kinetic models at a dose level of 0.01 mg/kg bw (Punt et al. 2009; Al-Subeihi et al. 2012; Alajlouni et al. 2016a; Al-Malahmeh et al. 2017), and (iii) the BMDL₁₀ values derived from *in vivo* tumor data for safrole, estragole, and methyleugenol (Miller et al. 1983), or by read-across from safrole for myristicin and apiol (Alajlouni et al. 2016a; Al-Malahmeh et al. 2017). Methyleugenol was taken as the reference compound (TEF = 1.0), expressing the concentrations of the other alkenylbenzenes in methyleugenol equivalents (Table 2).

Using these TEF values and the toxic equivalency approach (TEQ) the combined EDI values obtained vary in the range of 17.3–62.9 µg methyleugenol equivalents/kg bw per day (Table 4).

Using the EDI values thus obtained MOE values were calculated. To this end different BMDL₁₀ values were used for different approaches to obtain the MOE values. The BMDL₁₀ values for the individual alkenylbenzenes were taken from literature (van den Berg et al. 2011; Alajlouni et al. 2016a; Al-Malahmeh et al. 2017), and were used to calculate MOE values for intakes of each individual alkenylbenzene (Table 5). MOE values thus obtained were all below 10000. Assuming equal potency, the BMDL₁₀ of methyleugenol of 15.3 mg/kg bw per day (van den Berg et al. 2011), being a dominant alkenylbenzenes in the samples was compared with the combined EDI. The MOE values thus obtained varies between 351 and 1071 (Table 5). For the TEQ approach, the BMDL₁₀ of methyleugenol of

15.3 mg/kg bw per day (van den Berg et al. 2011) was compared to the EDI expressed in methyleugenol equivalents to calculate the MOE. When using the TEQ approach, MOE values ranged from 243 to 883.

Table 5. The MOE values obtained for samples containing several alkenylbenzenes. MOE values were calculated for each individual alkenylbenzenes, and based on combined exposure estimates either assuming equal potency, or based on a TEQ approach.

Sample no.	MOE individual alkenylbenzenes				MOE based on equal potency and BMDL ₁₀ of the highest level alkenylbenzene in the sample	MOE based on TEF
	methyleugenol	estragole	myristicin	apiol		
16	1263	3209			674	304
18	1166	355			1071	883
19	450		199		351	243
21	1028		221	2582	892	353

From the data thus obtained it is clear that the estimated daily consumption of the pesto sauces at 10 g basil per meal per day (based on wet weight) would result in MOE values that indicate a priority for risk management for all pesto sauces. It is of interest to evaluate what level of methyleugenol, amount of pesto sauce consumed and/or what exposure frequency would result in an MOE of 10000. To evaluate how these parameters could be modified to result in MOE values that would be of low priority for risk management, Figure 2 shows the relation between the level of methyleugenol detected in a pesto sauce sample and the amount of pesto sauce consumed that would result in MOE values of 10000 at a certain frequency of intake. For these calculations, it was assumed that Haber's rule would apply so the daily dose would be linearly dependent on the frequency of intake. The results presented indicate that daily intake of for example 30 g pesto sauce would be of low priority for risk management at a methyleugenol level of 3.6 µg/g, while weekly intake of 30 g would be of low concern at a methyleugenol value of 25.0 µg/g. The curves can also be used to derive that for the pesto

sauce samples of the present study with methyleugenol levels that vary from 99.3 to 3.6 $\mu\text{g/g}$ pesto sauce, the consumption of 1.1–29.8, 7.5–208, 15.1–416.5, and 32.4–892.5 g of pesto sauce on a daily basis, once a week, once every two weeks, and once a month, respectively, could be considered of low concern.

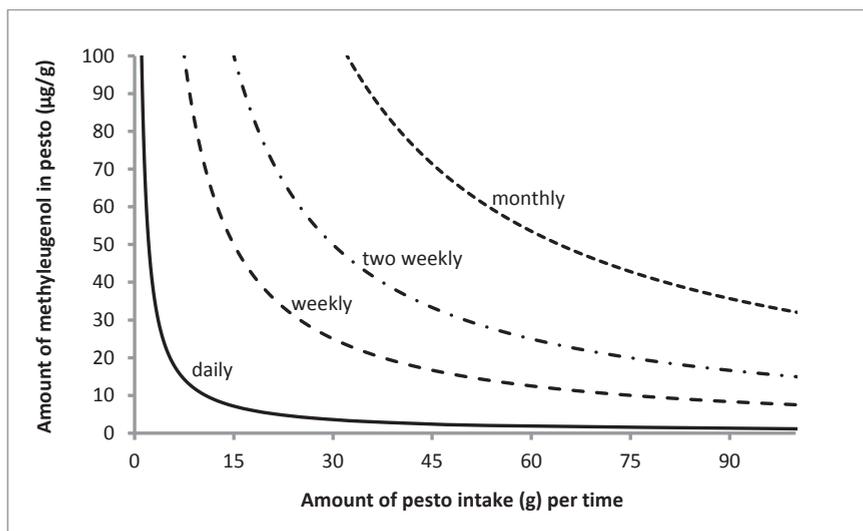


Figure 2. Relation between the methyleugenol content ($\mu\text{g/g}$) and daily pesto sauce consumption that would result in an MOE of 10000 upon daily (—), weekly (— —), two weekly (— • —), or monthly (••••) consumption. Combinations of methyleugenol levels and pesto sauce intake that result in values below the respective curves result in MOE values above 10000 and would be of low priority for risk management.

DISCUSSION

The aim of the present study was to make a more detailed analysis of the presence of methyleugenol and possible other alkenylbenzenes in basil-containing sauce of pesto and perform an associated risk assessment based on the MOE approach, taking into consideration the combined exposure to different alkenylbenzenes.

It is important to consider the variance in aromatic composition of basil at different growth stages from different areas. The methyleugenol content of the volatile oil from commercial

samples of sweet basil, and in the leaf, flower and stem for the same cultivar of sweet basil were in the range of 0–2.4 % (Tsai and Sheen 1987; Sheen et al. 1991; Lawrence et al. 1993). It was also reported that the content of methyleugenol in the essential oil of basil plants decreases from 100 % to 10 % as plant height and maturity increase (Miele et al. 2001). Different studies revealed different levels of estragole and methyleugenol in basil for different genotypes and as a function of region and harvesting. The concentrations in fresh basil amount 10.2–2029 $\mu\text{g/g}$ for estragole and 0.07–560 $\mu\text{g/g}$ for methyleugenol (Siano et al. 2003; Lee et al. 2005; Avila et al. 2009). For pesto sauce, studies showed different concentrations of estragole that varied between 0.05 and 19.3 $\mu\text{g/g}$, and of methyleugenol at values that varied between 0.01 and 52 $\mu\text{g/g}$ (Siano et al. 2003; Avila et al. 2009). The levels of methyleugenol determined in the pesto sauces of the present study amounted to 3.6–99.3 $\mu\text{g/g}$ pesto sauce, and of estragole, being present in only two out of the 32 pesto sauce samples analyzed, to 3.2 and 34.1 $\mu\text{g/g}$ pesto sauces. Based on these levels and taking the basil content (%) as indicated on the label of the pesto sauces into account, the level of methyleugenol in basil used to prepare the pesto sauce was estimated to vary between 22.4–310.3 $\mu\text{g/g}$. The determination of the levels of alkenylbenzenes in basil-containing sauce of pesto revealed that all the samples analyzed contained methyleugenol. In addition to methyleugenol, two of the samples contained estragole, while two other samples contained myristicin, and one sample contained both myristicin and apiol in addition to methyleugenol. Based on chemical analysis the EDI values could be determined assuming a body weight of 70 kg and intake of 10 g of basil (based on wet weight) per meal (Miele et al. 2001), provided by an amount of pesto sauce that could be calculated taking into account the basil content (%) in the pesto sauce as indicated on the label (Table 1). For samples containing only methyleugenol, the EDI values ranged from 3.2–44.3 $\mu\text{g/kg bw per day}$. These EDI values resulted in MOE values of 345–4781. Since several alkenylbenzenes were found in four of the pesto samples, and these

alkenylbenzenes show high similarity in structure, target organ, type of adverse effects and mode of action through formation of a DNA reactive 1'-sulfoxymetabolite contributing to formation of liver tumors, for these four samples a combined exposure assessment and subsequent risk assessment were performed. EDI values based on individual compounds amounted to 1.2–34.0 $\mu\text{g}/\text{kg}$ bw per day. These EDI values resulted in MOE values of 199–3209 for the individual levels of estragole, myristicin and apiole, indicating that these alkenylbenzenes add to the possible health risks. Assuming equal potency of the alkenylbenzenes, combined EDI values obtained ranged from 14.3–43.5 $\mu\text{g}/\text{kg}$ bw per day resulting in MOE values ranging from 351 to 1071. Estimation of the TEF values used in this study was based on *in vitro* and *in vivo* data. It is of interest to note that in an ideal situation TEF values would be derived from *in vivo* data. However in practice *in vitro* endpoints are also frequently used, for example in the case of TEF values derived for dioxins (Van den Berg et al. 2006) and, more recently, in the case of interim TEF values defined for pyrrolizidine alkaloids (Merz and Schrenk 2016). Using a TEF concept combined EDI values ranged from 17.5–59.7 μg methyleugenol equivalents/kg bw leading to MOE values ranging from 243 to 883. MOE values resulting from this combined exposure assessment were 1.5–5-fold lower than the MOE values obtained when considering the presence of only methyleugenol in these four samples, and are all below 10000. Combined risk assessment based on equal potency did not vary much from results obtained using the TEQ approach. This can be ascribed to the minor variation in potencies of the different alkenylbenzenes detected in the pesto samples. It is of interest to note that these MOE values refer to regular daily consumption of pesto which may be an overestimation of realistic human consumption. Levels of methyleugenol in pesto sauce samples and consumption frequency of pesto sauce directly impact the EDI and the resulting MOE values. The levels of methyleugenol detected in the pesto sauces of the present study vary between 99.3 and 3.6 $\mu\text{g}/\text{g}$ and would allow consumption of 1.1–29.8, 7.5–

208, 15.1–416.5, and 32.4–892.5 g of pesto sauce on a daily basis, once a week, once every two weeks, and once a month, respectively, to achieve MOE values above the 10000 safety limit for low priority for risk management. It is concluded that consumption of pesto sauces would only be of concern if consumed on a daily basis over longer periods of time. The results of the present paper reveal that pesto consumption does not always represent a cancer risk and provide insight into when this might be the case and under which level and frequency of consumption pesto consumption would not raise a concern.

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Chapter 5

Determination and risk assessment of naturally occurring genotoxic and carcinogenic alkenylbenzenes in nutmeg-based plant food supplements

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ABSTRACT

A risk assessment of nutmeg-based plant food supplements (PFS) containing different alkenylbenzenes was performed based on the alkenylbenzene levels quantified in a series of PFS collected via the online market. The estimated daily intake (EDI) of the alkenylbenzenes amounted to 0.3 to 312 $\mu\text{g kg}^{-1}$ body weight (bw) for individual alkenylbenzenes, to 1.5 to 631 $\mu\text{g kg}^{-1}$ bw when adding up the alkenylbenzene levels assuming equal potency, and to 0.4 to 295 $\mu\text{g kg}^{-1}$ bw when expressed in safrole equivalents using toxic equivalency factors (TEFs). The margin of exposure approach (MOE) was used to evaluate the potential risks. Independent of the method used for the intake estimate, the MOE values obtained were generally lower than 10000 indicating a priority for risk management. When taking into account that PFS may be used for shorter periods of time and using Haber's rule to correct for shorter than lifetime exposure it was shown that limiting exposure to only 1 or 2 weeks would result in MOE values that would be, with the presently determined levels of alkenylbenzenes and proposed uses of the PFS, of low priority for risk management (MOE > 10000). It is concluded that the results of the present paper reveal that nutmeg-based PFS consumption following recommendations for daily intake especially for longer periods of time raise a concern.

Keywords: margin of exposure (MOE); plant food supplement (PFS); nutmeg-based PFS; alkenylbenzenes; combined risk assessment; toxic equivalency factors (TEFs)

INTRODUCTION

Plant food supplements (PFS) are concentrated sources of dietary ingredients derived from various plants and herbal extracts. PFS are usually marketed in the form of capsules or powder and are supposed to supplement the normal diet. Regional regulations, consumer awareness, product availability and acceptance all affect the market for herbal supplements. The global herbal supplement market witnessed fast growth over the last decade and is expected to reach US\$112 billion by the end of the year 2020 (Strategyr, 2015), with Europe accounting for the largest share of the world market (Vargas-Murga et al., 2011). In the US, the supplement industry is approximately \$36.7 billion (Bradley, 2015). Online marketing of PFS has been increasing, and the sale online is expected to be of growing interest (Vargas-Murga et al., 2011). The need for stricter control and safety testing of PFS products is becoming more vital to protect consumers against adverse health effects. Some PFS may consist (in part) of botanicals and/or botanical preparations that contain compounds that are of concern for human health. Previously, some general concerns related to the quality and safety of botanical preparations such as PFS were expressed by the European Food Safety Authority (EFSA), and a compendium listing botanicals known to contain naturally occurring substances of possible concern for human health when used in food and food supplements was published (EFSA, 2012a). Specific concerns were expressed over the presence of naturally occurring toxic compounds (EFSA, 2004). The group of alkenylbenzenes, for example, contains compounds such as estragole, methyleugenol, safrole, elemicin, myristicin and apiol (Figure 1), that are genotoxic and carcinogenic, and may be present in botanicals like basil, nutmeg, tarragon and fennel (SCF, 2001a, b, 2002; Rietjens et al., 2008). Nutmeg is derived from several species of trees in the genus *Myristica*, mostly from *Myristica fragrans*, and known to contain, in addition to methyleugenol and safrole, especially

another alkenylbenzene, namely myristicin (van den Berg et al., 2011; Britannica, 2016). In 2001, The Scientific Committee on Food (SCF) concluded that the structural analogues, methyleugenol, estragole, and safrole are genotoxic and carcinogenic and indicated the need for a reduction in exposure and restrictions in the levels of use (SCF, 2001a, b, 2002). Rodent studies performed at high dose levels provided sufficient evidence of the hepatocarcinogenicity of methyleugenol, estragole and safrole, displaying similar characteristics regarding mode of action and tumor formation (Phillips et al., 1981; Miller et al., 1983; Randerath et al., 1984; Wiseman et al., 1985; NTP, 2000; Zhou et al., 2007; Kobets et al., 2016). EC Regulation 1334/2008, which became effective in January 2011, prohibits the addition of methyleugenol, estragole and safrole to foods and restricts their concentration in certain compound food as consumed to which flavorings and/or food ingredients with flavoring properties have been added (European Commission, 2008).

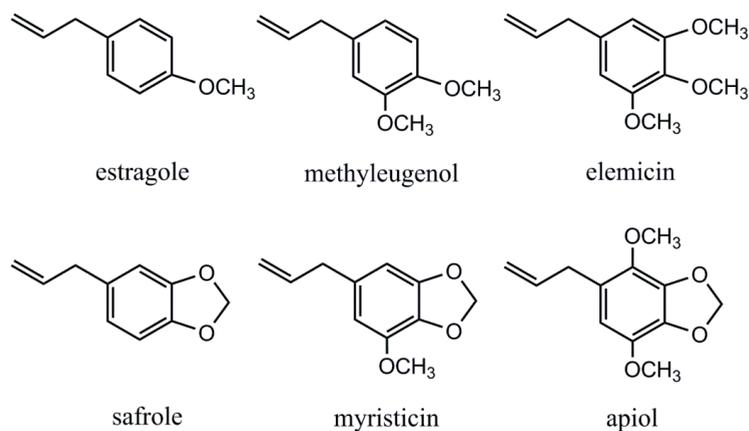


Figure 1. The structural formulas of the alkenylbenzenes estragole, methyleugenol, elemicin, safrole, myristicin and apiol

A major issue in the assessment of the potential risks of human exposure to these genotoxic and carcinogenic compounds via the use of alkenylbenzene-containing PFS is how to perform this assessment (EFSA, 2005). For compounds that are both genotoxic and carcinogenic, the EFSA recommended the Margin of Exposure (MOE) approach to evaluate the priority for risk management actions (EFSA, 2005, 2009a). The MOE compares the exposure levels causing malignant tumors in experimental animals with dietary intake estimates (EDI) in humans, considering differences in consumption patterns. To obtain the MOE, EFSA recommends the use of the BMDL₁₀ (the lower confidence limit of the benchmark dose resulting in 10% extra cancer incidence) (EFSA, 2009b). The BMDL₁₀ is a standardized reference point derived, by mathematical modelling, from the animal data within the observed range of experimental data (EFSA, 2009a). Using the MOE approach, van den Berg et al. (2011) revealed that 25 out of 28 PFS samples containing botanicals including basil, fennel, saffron, nutmeg, cinnamon, and calamus had alkenylbenzenes at levels that would result in estimated daily intakes that would give rise to MOE values below 10000, indicating a priority for risk management. The aim of the present study was to make a more detailed and extended analysis of the presence of different alkenylbenzenes in nutmeg-based PFS and perform an associated risk assessment based on the MOE approach, taking into consideration the possible combined exposure to different alkenylbenzenes. To this end, the level of alkenylbenzenes in various nutmeg-containing PFS was quantified providing the basis for the risk assessment. In this risk assessment, combined exposure to different alkenylbenzenes was also taken into account. For this group of botanical ingredients combined exposure can be evaluated by dose addition, which is considered appropriate for mixtures with components acting by a similar mode of action, at a similar target organ, i.e. causing liver carcinogenicity (EPA, 1986a, 1988, 2000). This implies that the response

to the mixture can be predicted by summing the doses of the components, if needed, after adjusting for the differences in potencies using toxic equivalency factors (TEFs) (ATSDR, 2004).

MATERIALS AND METHODS

Nutmeg-based PFS samples and chemicals

Nutmeg-based PFS samples were purchased online. Table 1 presents an overview of the collected samples and their major characteristics as indicated on the label of each product.

Methyleugenol, myristicin, and safrole (purity, > 97%), methanol (HPLC grade) and acetonitrile (LC/MS grade) were supplied by Sigma-Aldrich (Zwijndrecht, Netherland). Elemicin was obtained from Synchem OHG (Felsberg, Germany). Nano pure water was obtained from a Barnstead Nanopure Type I ultrapure water system. Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium). Acetonitrile (ACN) (LC/MS grade) was purchased from Biosolve BV (Valkenswaard, The Netherlands). Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany).

Methanol extracts

All nutmeg-based PFS samples were extracted using methanol based on the method described by Gursale et al. (2010) with minor modifications. In short, 0.5 g of each PFS sample was sonicated in an ultrasonic bath for 10 min followed by maceration for 12-hr at 50°C with 80 ml of methanol in a closed glass vessel. Finally, an aliquot of the extract solution was centrifuged at 13000×g for 5min, and stored at 20°C until Ultra Performance Liquid Chromatography (UPLC) analysis. Samples were extracted and analysed on UPLC in three independent experiments. The

accuracy of the developed method was assessed using a recovery study and standard deviations of the replicates. For recovery studies, pure standards of commercially available alkenylbenzenes were added in two different quantities (final concentration of 5 and 10 μM) to 0.5 g of two PFS samples (sample 5 and 8) which covers the whole range of analyses. Samples were prepared and analyzed as described above to quantify the alkenylbenzene levels enabling determination of the recovery. The extraction capacity linearity was demonstrated by analysing a range of weight of the sample to a final volume of the solution ratio of 0.3 % up to 1 % (w/v).

UPLC analysis

For the determination of the alkenylbenzene level in the extracts, 3.5 μl of each sample was subjected to UPLC analysis. The UPLC system consisted of a Waters (Milford, MA, USA) Acquity solvent manager, sample manager and photodiode array detector. Chromatographic conditions for compound separation were achieved using an ACQUITY UPLC BEH C18 1.7 μm column, 2.1 \times 50 mm. The column oven was set at 35°C and the sample compartment was set at 10°C. An isocratic gradient was maintained for the separation using a mixture of acetonitrile (ACN) and ultrapure water containing 0.1 % (v/v) trifluoroacetic acid (TFA). The mobile phase consisted of 40 % acetonitrile for 4min, which was the time needed to separate the alkenylbenzenes of interest. During the whole run, the flow rate was 0.6 ml min^{-1} . Under these conditions, the retention times for elemicin, methyleugenol, safrole, and myristicin were 1.4, 2.1, 2.2 and 2.4 min, respectively. Identification of the alkenylbenzenes was achieved by comparing the UV spectrum and retention time of the peak to the UV spectrum and the peak retention time of commercially available reference compounds. Quantification was done by comparison of the area of the alkenylbenzene peak to that of the calibration curve of the reference compound

Table 1. Nutmeg-based PFS samples used in the present study and their major characteristics. Information provided was derived from the product labels unless stated otherwise

PFS sample	Description	Ingredients	Recommendation for daily intake
1	Ayurvedic nutmeg herbal supplement "complementary or alternative medicine"	Nutmeg powder (<i>Myristica fragrans</i>). Capsule content: 330 mg	2 capsules (660 mg)
2	Dietary supplement	Nutmeg powder (<i>Myristica fragrans</i>). Capsule content: 750 mg	1-5 capsules (3750 mg)
3	Dietary supplement	Nutmeg powder (<i>Myristica fragrans</i>). Capsule content: 600 mg	2 capsules, (1200 mg) with water at meal time
4	Nutmeg herbal supplement "complementary or alternative medicine"	Nutmeg powder (<i>Myristica fragrans</i>). Capsule content: 360 mg	1-3 capsules (360 mg)
5	100% pure nutmeg Therapeutic grade essential oil supplement	Essential oil of nutmeg . Every drop is around 72 mg	Dilute 1 drop with 1 drop of V-6™ or olive oil. Put in a capsule and takes up to three times daily or as needed (216 mg)
6	Ayurvedic invigorates male sexual vitality herbal supplement	Blend of cinnamon camphora, nutmeg (<i>Myristica fragrans</i>), hygrophila auriculata, tribulus terrestris, withania somnifera, asparagus recemosus, cardamom, dioscorea sativa, shilajit, processed silica. Capsule content: 350 mg	4 capsules, 15-30 minutes after meals, daily over a period of at least 2-3 months (1400 mg)
7	Weight loss formula and clean sensory experience, dietary supplement.	Super xtreme supergenic complex of: green coffee bean extract (52.6%), caffeine anhydrous (17.8%), nutmeg seed extract (<i>Myristica fragrans</i>) (13.2%), sage leaf powder (salvia officinalis) (6.5%), alpha lipoic acid (3.3%), cocoa seed extract (theobroma cacao) (3.3%), green tea leaf and shoot extract (camellia sinensis) (3.3%). Capsule content: 760 mg	3 capsules (2280 mg)
8	Nutmeg herbal supplement	Nutmeg powder (<i>Myristica fragrans</i>). Capsule content: 100 mg	6 capsules (600 mg)
9	Nutmeg herbal supplement	Nutmeg powder (<i>Myristica fragrans</i>). Capsule content: 900 mg	2 capsules (1800 mg)
10	Organic nutmeg herbal supplement seed capsules	Nutmeg powder (<i>Myristica fragrans</i>). Capsule content: 465 mg	2 capsules (930 mg)
11	Herbal supplement, Vigor care for woman, for female libido support, help maintain normal sexual desire	Mix of: ashwagandha (root), nutmeg (aril) , lodh tree (bark), cyperus (tuber), spanish pellitory (root), ginger (rhizome). Capsule content: 820 mg	2 capsules (1640 mg)
12	Organic nutmeg seed capsules, herbal dietary supplement	Organic nutmeg seed, pure herb (<i>Myristica fragrans</i>). Capsule content: 470 mg	1-2 capsules, 2-3 times (940 mg)
13	Nutmeg herbal supplement	Nutmeg powder (<i>Myristica fragrans</i>). Capsule content: 400mg	1-2 capsules (400 mg)

determined using UPLC with photodiode array detection (UPLC/PDA) at a wavelength 201 nm for elemicin and methyleugenol, and a wavelength 210 nm for myristicin and safrole.

Determination of EDI of alkenylbenzenes resulting from the use of nutmeg-based PFS

The EDI of alkenylbenzenes resulting from the use of the nutmeg based PFS was determined based on the alkenylbenzene content as determined in the present study (see Results) and a body weight of 70 kg, the default value for adult body weight proposed by EFSA (EFSA, 2012b). Daily consumption of nutmeg-based PFS was also based on the recommendation for daily intake provided by the manufacturer and the amount of the material stated on the label to be present in each capsule or drop (Table 1). First, the EDI was calculated for individual compounds for all the samples. As several samples were found to contain more than one alkenylbenzene, and the different alkenylbenzenes show similarity in structure, target organ, type of adverse effects and mode of action through formation of a DNA reactive 1'-sulfoxymetabolite contributing to formation of liver tumors, a combined exposure assessment and subsequent risk assessment were also performed for those samples. To this end two approaches were used; summing up the EDIs of the alkenylbenzenes assuming equal potency, and a so-called toxic equivalency (TEQ) approach calculating the EDI values of the alkenylbenzenes using their TEF relative to safrole (Alajlouni et al., 2016b). In this study, safrole was taken as the reference compound (TEF = 1.0), enabling expression of the overall alkenylbenzene concentration and EDI in safrole equivalents. TEF values for the different alkenylbenzenes for the TEQ approach need to be defined, which was done by calculating an average of three endpoints. The first one was the slope of the dose–response curve for DNA-adduct formation in female CD-1 mice liver upon exposure to different alkenylbenzenes (Randerath et al., 1984). The second one, was the *in vivo* level of formation of

the hepatocarcinogenic 1'-sulfoxymetabolite of the different alkenylbenzenes predicted using human physiologically based kinetic (PBK) models at a dose level of 0.01mg kg⁻¹ bw (Punt et al., 2009; Al-Subeihi et al., 2012; Martati et al., 2012; van den Berg et al., 2012; Alajlouni et al., 2016a; Al-Malahmeh et al., 2017). A third value was defined based on the BMDL₁₀ values derived from *in vivo* tumor data for safrole, estragole and methyleugenol (Miller et al., 1983), and by read-across from safrole for myristicin and apiol (Alajlouni et al., 2016a; Al-Malahmeh et al., 2017), or by read-across from methyleugenol and estragole for elemicin (van den Berg et al., 2012). The TEF values for the different alkenylbenzenes defined by the three approaches relative to safrole were subsequently averaged to define the final TEF values (Alajlouni et al., 2016b). Using these TEF values, the EDI of alkenylbenzenes in the nutmeg-based PFS were expressed in µg safrole equivalents kg⁻¹ bw per day.

Calculation of the margin of exposure

Risk assessment of consumption of the nutmeg-based PFS samples was performed using the MOE approach (EFSA, 2005). For the individual alkenylbenzene approach, the BMDL₁₀ values of the individual compounds were used (van den Berg et al., 2012; Alajlouni et al., 2016a; Al-Malahmeh et al., 2017), and then compared with the EDIs of the alkenylbenzenes resulting from the use of nutmeg-based PFS. For the equal potency assumption approach, the BMDL₁₀ value of myristicin, the major alkenylbenzene in most of the samples, was used and compared to the sum of the EDIs. And finally, for the TEQ approach, the BMDL₁₀ of the reference compound safrole was compared to the EDI expressed in safrole equivalents.

RESULTS

Table 2 presents the level of the different alkenylbenzenes found in the PFS samples. In eight PFS samples, four different alkenylbenzenes were detected including elemicin, methyleugenol, myristicin and safrole. In PFS 6, elemicin and myristicin were detected. Elemicin, myristicin, and safrole were detected in PFS 10, and elemicin, methyleugenol, and safrole in PFS 12. PFS 7 and PFS 11 did not contain a detectable level of any of the alkenylbenzenes. Table 2 also presents the EDI of the individual alkenylbenzenes obtained for these samples when considering the recommended dose regimen. The values obtained varied from 0.3 to 312 $\mu\text{g kg}^{-1}$ bw per day with myristicin being the major alkenylbenzene. Figure 2 presents the relative contribution of the different alkenylbenzenes to the total alkenylbenzene content of the different PFS and shows that myristicin makes up 18.5 to 85.1 % of the sum of all alkenylbenzenes, followed by elemicin making up 3.7 to 81.5%, then safrole making up 8.8 to 25.1%, and finally methyleugenol being the minor alkenylbenzene making up 2.4 to 21.0%. As several alkenylbenzenes were found in the samples, and because these alkenylbenzenes are known to display similarity in structure, target organ, type of adverse effects and mode of action through the formation of a DNA reactive 10-sulfoxymetabolite contributing to the formation of liver tumors, a combined exposure and risk assessment were also performed. To this end, the combined exposure was estimated in two ways, assuming either equal potency or using a so-called TEQ approach. When assuming equal potency of the different alkenylbenzenes the combined EDI can be calculated by adding up the EDIs of the different alkenylbenzenes. In a second approach, the combined exposure was calculated in safrole equivalents taking the relative potency of the different alkenylbenzenes into account. Using the equal potency approach, the combined EDI values obtained vary in the range

of 1.5 to 631 $\mu\text{g kg}^{-1}$ bw per day. Using TEF values and the TEQ the combined EDI values obtained vary in the range of 0.4 to 295 $\mu\text{g safrole equivalents kg}^{-1}$ bw per day (Table 3).

Using the EDI values thus obtained MOE values were calculated. To this end, different BMDL₁₀ values were used for different approaches to obtain the MOE values. The BMDL₁₀ values for the individual alkenylbenzenes were taken from literature (van den Berg et al., 2012; Alajlouni et al., 2016a; Al-Malahmeh et al., 2017) and were used to calculate MOE values for intakes of each alkenylbenzene (Table 4). MOE values thus obtained were all below 10000 except for elemicin in samples 4 and 6, with MOE values amounting to 10729 and 25877, respectively. Assuming equal potency, the BMDL₁₀ of myristicin of 1.9 mg kg^{-1} bw per day (Al-Malahmeh et al., 2017), was compared with the combined EDI, because in most cases myristicin was the major alkenylbenzene detected in the samples. The MOE values thus obtained vary between 3 and 1309 (Table 4). For the TEQ approach, the BMDL₁₀ of safrole of 1.9mg kg^{-1} bw per day (van den Berg et al., 2012) was compared with the EDI expressed in safrole equivalents to calculate the MOE. When using the TEQ approach, MOE values ranged from 6 to 5020 (Table 4). Comparison of the MOE values obtained when using the combined exposure reveals that the values tend to increase when using the TEF concept. This is because the TEF for myristicin, the major alkenylbenzene detected in the samples, is lower than 1.0 resulting in lower EDI and higher MOE values. In spite of these differences, the priority for risk management for the PFS does not vary because the MOE remains below 10000 (in most cases even below 1000). From the data thus obtained it is clear that the EDI resulting from the daily use of the nutmeg-based PFS would generally result in MOE values that indicate a priority for risk management, independent of the way in which the EDI and the MOE were calculated.

Table 2. Level of alkenylbenzenes in nutmeg-based PFS samples, and EDI estimate for the individual alkenylbenzenes. ND; not detected, NA; not applicable.

PFS sample	Alkenylbenzene level in PFS samples ^a						EDI										
	Elemicin		Methyleugenol		Myristicin		Safrole		Elemicin		Methyleugenol		Myristicin		Safrole		
	µg/g	µmol/g	µg/g	µmol/g	µg/g	µmol/g	µg/g	µmol/g	µg/kg bw/day	µg/kg bw/day	µg/kg bw/day	µg/kg bw/day	µg/kg bw/day	µg/kg bw/day	µg/kg bw/day	µmol/kg bw/day	
1	660	508 ± 43	2.4	623 ± 22	3.5	4208 ± 403	21.9	896 ± 74	5.5	4.8	0.023	5.9	0.033	39.7	0.206	8.4	0.052
2	3750	5515 ± 699	26.5	492 ± 116	2.8	4490 ± 533	23.4	1274 ± 65	7.9	295.5	1.419	26.3	0.148	240.5	1.251	68.3	0.421
3	1200	227 ± 15	1.1	120 ± 6	0.7	487 ± 82	2.5	280 ± 48	1.7	3.9	0.019	2.1	0.012	8.3	0.043	4.8	0.030
4	360	555 ± 295	2.7	684 ± 43	3.8	8982 ± 1039	46.7	1663 ± 172	10.3	2.9	0.014	3.5	0.020	46.2	0.240	8.5	0.053
5	220	4448 ± 79	21.4	2811 ± 136	15.8	101125 ± 5183	526.1	10494 ± 356	64.7	13.7	0.066	8.7	0.049	312.0	1.623	32.4	0.200
6	1400	59 ± 13	0.3	ND	ND	13 ± 3	0.1	ND	ND	1.2	0.006	NA	NA	0.3	0.001	NA	NA
7	2280	ND	ND	ND	ND	ND	ND	ND	ND	NA	NA	NA	NA	NA	NA	NA	NA
8	600	2471 ± 136	11.9	1441 ± 118	8.1	4934 ± 601	25.7	1189 ± 147	7.3	21.2	0.102	12.3	0.069	42.3	0.220	10.2	0.063
9	1800	1018 ± 74	4.9	84 ± 31	0.5	464 ± 66	2.4	182 ± 59	1.1	26.2	0.126	2.2	0.012	11.9	0.062	4.7	0.029
10	930	9655 ± 500	46.4	ND	ND	7028 ± 788	36.6	2115 ± 839	13.0	128.3	0.616	NA	NA	93.4	0.486	28.1	0.173
11	1640	ND	ND	ND	ND	ND	ND	ND	ND	NA	NA	NA	NA	NA	NA	NA	NA
12	940	5680 ± 222	27.3	501 ± 24	2.8	ND	ND	1376 ± 48	8.5	76.3	0.366	6.7	0.038	NA	NA	18.5	0.114
13	400	2612 ± 343	12.5	2814 ± 184	15.8	6574 ± 24	34.2	1398 ± 87	8.6	14.9	0.072	16.1	0.090	37.6	0.195	8.0	0.049

^a Average ± SD of 3 separate preparations/extraction

^b Calculated using the formula: $EDI (\mu\text{g}/\text{kg}\cdot\text{bw}/\text{day}) = \frac{\text{alkenylbenzene level } (\mu\text{g}/\text{g})}{\text{adult body weight (70 kg)}} \times \text{recommended daily intake (g)}$

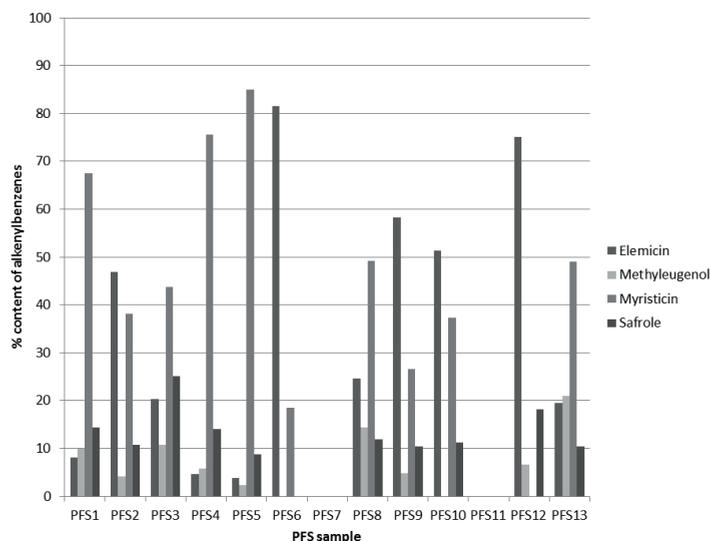


Figure 2. Level of the different alkenylbenzenes in the nutmeg-based plant food supplements (PFS) samples expressed as percentage of the total amount of alkenylbenzenes.

It is of importance; however, to consider that often use of PFS will not be for a lifetime but for shorter periods of time and how this may affect the risk assessment. There is no generally established method for taking a shorter than lifetime exposure into account, but one could consider using Haber's rule. Assuming that Haber's rule would apply, the toxic effect would vary linearly with the time of exposure and the concentration (i.e. $C \times T = k$, where C is concentration or dose, T is time of exposure, and k is a constant toxic response for the specific substance) (Doull and Rozman, 2000). It is of interest to evaluate, using Haber's rule, what level of alkenylbenzenes, what amount of nutmeg-based PFS consumed and/or what exposure frequency would result in an MOE of 10000 and be of low priority for risk management. To this end, Figure 3 shows the relation between the total recommended daily intake of nutmeg-based PFS and the total level of alkenylbenzene in nutmeg-based PFS samples that would result in

MOE values of 10000 assuming equal potency but different periods of use, including life time (estimated life expectancy of 75 years) (Felter et al., 2011), 4, 2, or 1 week in a life time. The results presented indicate that the daily recommended consumption range of samples that contain 216 to 3750 mg nutmeg-based PFS, the range of recommended daily intake (Table 1) would be of low priority for risk management at a total alkenylbenzene level of 61.6 to 3.6 $\mu\text{g g}^{-1}$ for life-time exposure. Intake of the recommended daily consumption of the nutmeg-based PFS, for limited periods of time, for example, 4, 2 and 1 week, would be of low priority for risk management when the PFS would contain total alkenylbenzene levels of 60032 to 3458, 120069 to 6916, and 240139 to 13832 $\mu\text{g g}^{-1}$, respectively.

Table 3. Combined EDI values for PFS samples, tested positive for the presence of alkenylbenzenes, assuming equal potency and using a TEQ approach

PFS sample	EDI	
	Equal potency ^a ($\mu\text{g}/\text{kg bw day}$)	TEQ ^b ($\mu\text{g safrole equivalents}/\text{kg bw}$)
1	58.8	39.3
2	631	295
3	19.1	12.3
4	61.1	41.8
5	367	246
6	1.5	0.4
8	86.0	49.9
9	45.0	18.5
10	250	112
12	102	36.0
13	76.6	46.0
^a Calculated using the formula; $EDI (\mu\text{g}/\text{kg bw day}) = \sum_i EDI$		
^b Calculated using the formula; $EDI (\mu\text{g}/\text{kg bw day}) = \sum_i (EDI \times TEF)_i$		
<u>Average TEF values \pmSD (Alajlouni et al. 2016b)</u>		
Safrole	1.00 (the reference compound)	
Myristicin	0.66 \pm 0.39	
Methyleugenol	0.67 \pm 0.46	
Elemicin	0.17 \pm 0.16	

The curves can also be used to derive that for the nutmeg-based PFS of the present study with total alkenylbenzene levels that vary from 73 to 118878 $\mu\text{g g}^{-1}$ nutmeg-based PFS, daily consumption for a lifetime should be limited to 182 to 0.1 mg of the nutmeg-based PFS to be of low concern. For shorter periods of time for example 4, 2, and 1 week, the maximum allowance of consumption of the nutmeg-based PFS for the levels of alkenylbenzenes quantified in this study would be of low priority for risk management when the PFS would contain 177630 to 109, 355274 to 218, and 710548 to 436 mg nutmeg-based material. This result also indicates that at the current levels of alkenylbenzenes in nutmeg-based PFS, their proposed uses would be of concern especially for people who use these PFS frequently during longer periods of time.

Table 4. MOE values for PFS, tested positive for presence of alkenylbenzenes, based on EDI values for individual alkenylbenzenes, and on combined EDI values calculated assuming equal potency and using a TEQ approach

PFS Sample	MOE					
	Individual				Equal potency ^a	TEQ ^b
	Elemicin	Methyleugenol	Myristicin	Safrole		
1	6386	2604	48	225	32	48
2	104	581	8	28	3	6
3	7870	7425	228	396	100	154
4	10729	4349	41	222	31	45
5	2229	1764	6	59	5	8
6	25877	NA	7072	NA	1309	5020
8	1445	1239	45	186	22	38
9	1169	7062	159	406	42	103
10	239	NA	20	68	8	17
12	401	2272	NA	103	19	53
13	2050	952	51	238	25	41

^a Calculated using the BMDL₁₀ of myristicin, the major alkenylbenzene in most of the samples

^b Calculated using the BMDL₁₀ of safrole, (the reference compound)

To further illustrate how short-term exposure would affect F4 the MOE values, Figure 4 presents a comparison between MOE values obtained for combined exposure during lifetime and 4weeks. Figure 5 shows the number of weeks of daily consumption of nutmeg-based PFS samples analysed in this study that would result in a MOE of 10000 indicating a low priority for risk management.

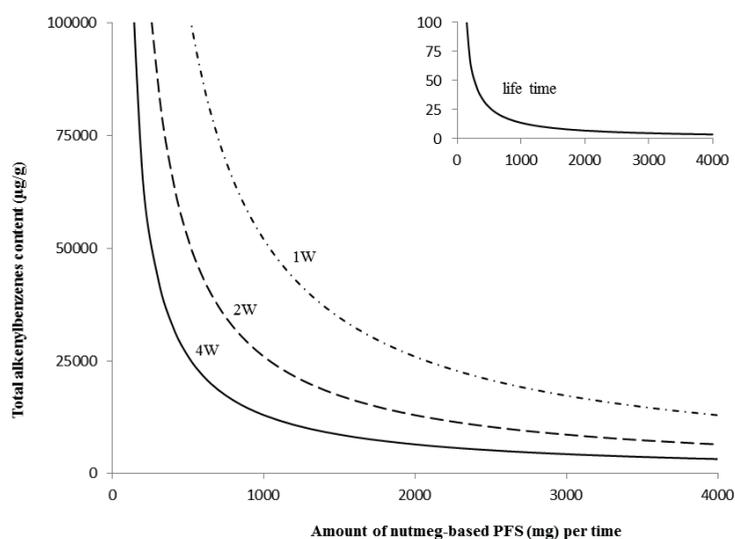


Figure 3. Relation between the total alkenylbenzene content ($\mu\text{g g}^{-1}$) in a nutmeg-based PFS and the recommended daily intake of the PFS (mg), that would result in an MOE of 10000 upon daily consumption for a lifetime (insert), 1 (—•—), 2 (---), and 4 weeks (—). The calculations assumed combined exposure and equal potency of the different alkenylbenzenes and uses the BMDL_{10} of myristicin, the major alkenylbenzene detected in most PFS. Combinations of total alkenylbenzene levels and nutmeg-based PFS intake that result in values below the respective curves result in MOE values above 10000 and would be of low priority for risk management. W; week.

Assuming both approaches, the equal potency and the TEQ, the longest time of daily combined exposure that would make the risk of low priority for risk management was for PFS 6 (the sample with the lowest EDI; $1.5 \mu\text{g kg}^{-1} \text{ bw day}$) and amounted to 511 and 1958 weeks, and the shortest time of daily combined exposure to make the risk of low priority was for PFS 2 (the sample with the highest EDI; $631 \mu\text{g kg}^{-1} \text{ bw day}$) amounting to 1 and 3 weeks, respectively.

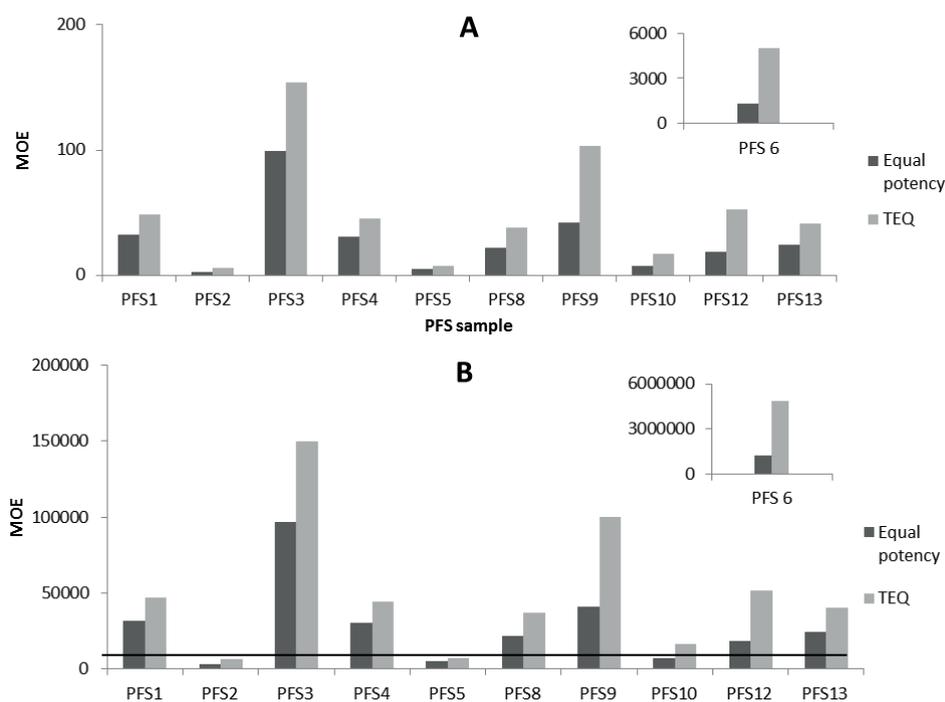


Figure 4. MOE values for the combined exposure assuming equal potency of the alkenylbenzenes and using the BMDL_{10} value of myristicin (black bars), and combined exposure using the TEQ approach based on safrrole equivalents and using the BMDL_{10} value of safrrole (grey bars), assuming life time intake (A), and 4 weeks intake (B). The black horizontal line in Figure B presents the MOE of 10000. Note the difference in the Y axis of Figure A and B. The values for PFS 6 are presented in the insert at the right top of the graph.

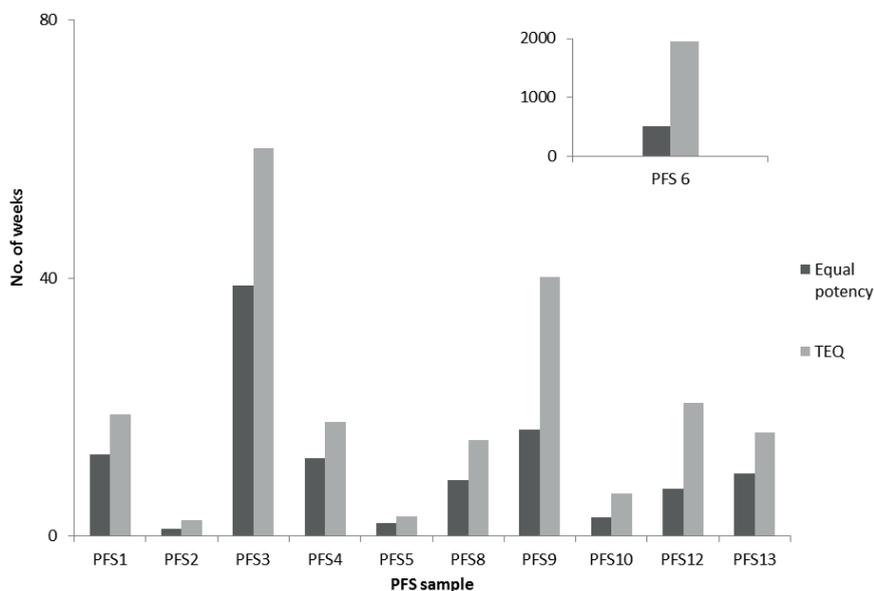


Figure 5. The number of weeks needed that would result in an MOE of 10000 upon daily consumption of nutmeg-based PFS samples analysed in the present study. The calculations assumed combined exposure and equal potency of the different alkenylbenzenes using the $BMDL_{10}$ of myristicin, the major alkenylbenzene detected in most PFS (black bars), and combined exposure using the TEQ approach based on safrole equivalents and using the $BMDL_{10}$ value of safrole (grey bars), also taking into account the total alkenylbenzene content ($\mu\text{g g}^{-1}$) of the nutmeg-based PFS obtained in the present study and the recommended daily intake of the PFS (mg) for the analysed samples. The values for PFS 6 are presented in the insert at the right top of the graph.

DISCUSSION

The aim of the present study was to make a more detailed and extended analysis of the presence of different alkenylbenzenes in nutmeg-based PFS and perform an associated risk assessment based on the MOE approach, taking into consideration the possible combined exposure to different alkenylbenzenes. Given that the different alkenylbenzenes act by a similar mode of action, at a similar target organ causing liver carcinogenicity (EPA, 1986a, 1988, 2000), combined exposure can be evaluated by dose addition; predicting the response to the mixture by adding up the doses of the components considering equal potency, and/or after adjusting for the differences in potencies, using the TEQ approach (ATSDR, 2004). PFS samples analyzed were made of nutmeg powder alone or from nutmeg combined with material from other botanicals (Table 1). Nutmeg is obtained from the nutmeg tree, *Myristica frangans* Houtt (*Myristicaceae*). The nutmeg seeds contain 5–15 % essential volatile oil and 24–40 % nutmeg butter (Forrest and Heacock, 1972). The relative percentages of the different components and the alkenylbenzenes vary depending on the geographical origin of the nutmeg and length of storage. The compounds that have been isolated and identified in the essential volatile oil were terpenes, monoterpene alcohol, a sesquiterpene, terpinic esters, acids, aromatic hydrocarbons, and our focus in this study; the aromatic ethers (myristicin, safrole, methyleugenol, and elemicin) (FAO, 1994). Levels of myristicin, elemicin, safrole, and methyleugenol detected in the nutmeg-based PFS samples analysed in the present study varied from 13 to 101124, from 59 to 9655, from 279 to 10494, and from 84 to 2813 $\mu\text{g g}^{-1}$ nutmeg-based PFS, respectively, with levels of these alkenylbenzenes making up 26.5 to 85.1 %, 3.7 to 81.5 %, 8.8 to 25.1 %, 2.4 to 21.0 % of the total alkenylbenzene content (Figure 2), with myristicin often being the major one detected, followed by elemicin and safrole, and methyleugenol. Literature data indicate that myristicin,

safrole and elemicin may account for 3.86 to 12.7 %, 0.53 to 3.42 % and 0.02 to 2.36 %, respectively of the nutmeg oil samples (IPCS, 1997). The wide variation in the alkenylbenzenes content of the different PFS may be caused by the variability between the quality (differences in composition) and quantity of nutmeg oil present in the various plants, depending on plant species, culture conditions, harvesting conditions and processing methods (Smith et al., 2002). Based on the alkenylbenzenes contents detected in the present study and the use levels of the respective PFS recommended on the labels, EDI values calculated for individual alkenylbenzenes ranged from 0.3 to 312 $\mu\text{g kg}^{-1}$ bw per day. Using the respective BMDL_{10} values these EDI values resulted in MOE values mostly below 10000 ranging from 6 to 7870, except for sample 4 and 6 for which the MOE value for elemicin of 10729 and 25877, respectively, exceeded the 10000 safety limit. A combined exposure assessment and subsequent risk assessment were performed for those PFS in which more than one alkenylbenzene was detected. Assuming equal potency of the alkenylbenzenes, combined EDI values obtained ranged from 1.5 to 631 $\mu\text{g kg}^{-1}$ bw per day resulting in MOE values ranging from 3 to 1309 for the samples in which more than one alkenylbenzenes was detected. Using a TEQ concept with TEF values based on *in vitro* and *in vivo* data (Miller et al., 1983; Randerath et al., 1984; Punt et al., 2009; Al-Subeihi et al., 2012; Martati et al., 2012; van den Berg et al., 2012; Alajlouni et al., 2016a; Al-Malahmeh et al., 2017), combined EDI values ranged from 0.4 to 295 μg safrole equivalents kg^{-1} bw leading to MOE values ranging from 6 to 5020. MOE values resulting from both these combined exposure assessments were up to 5-fold lower than the MOE values obtained when considering individual exposure to alkenylbenzenes. The MOE values obtained for the combined risk assessment based on the TEQ concept were higher than the ones obtained when assuming equal potency. This can be ascribed to the fact that the TEF values of the

alkenylbenzenes present in the PFS, including myristicin, elemicin, and methyleugenol, are lower than that of safrole leading to lower EDI values when expressed in safrole equivalents and higher MOE values.

It is of interest to note that these MOE values refer to regular daily consumption of nutmeg-based PFS during a whole lifetime which may be an overestimation of realistic human consumption.

Levels of alkenylbenzenes in nutmeg-based PFS samples and consumption frequency directly impact the EDI and the resulting MOE values. Using Haber's rule to correct for shorter than lifetime exposure it was also shown that limiting exposure to only one or two weeks could result in MOE values that would be, with the presently detected levels of alkenylbenzenes and proposed uses of the PFS, higher than 10000 and thus of low priority for risk management.

Use of Haber's Rule for products consumed for shorter periods of time may prevent overestimation of the actual risk to human health that may occur if MOE values are calculated assuming lifetime exposure. Applying Haber's Rule, however, is not a generally accepted approach when using the MOE for risk assessment of exposure to genotoxic carcinogens. Felter et al. (2011) was the first to propose use of Haber's Rule for assessing the risk from less-than lifetime exposure to carcinogens. It is important to acknowledge the assumptions underlying this methodology, which include the fact that chemical-specific carcinogenicity data should be available and that the data support a linear dose-response relationship (Felter et al., 2011). Whether such a linear dose-response relationship would apply to the dose-dependent induction of liver tumors by the alkenylbenzenes remains to be established and may depend on the mode of action underlying the carcinogenicity. This mode of action proceeds by cytochrome P450-catalyzed hydroxylation to a 1'-hydroxymetabolite, followed by sulfotransferase (SULT)-

mediated conversion to a 1'-sulfoxymetabolite (Phillips et al., 1981). This metabolite is unstable and reacts with DNA. Studies using PBK modelling revealed that the bioactivation of alkenylbenzenes to their ultimate carcinogenic 1'-sulfoxy metabolites is linear with the dose, from dose levels as low as levels of realistic human dietary intake up to dose levels as high as the BMD₁₀ inducing liver tumors in rodent bioassays (Punt et al., 2009; Rietjens et al., 2010; Al-Subeihi et al., 2012; Martati et al., 2012). The mutagenic potential of alkenylbenzenes DNA adducts has not been extensively quantified so far. Herrmann et al. (2012) investigated the mutagenic potential of sulfate conjugates of 1'-hydroxymethyleugenol and showed that different isomeric hydroxylated metabolites of methyleugenol are mutagenic in an Ames test using *Salmonella typhimurium* TA100 strains expressing different sulfotransferase enzymes. This suggests that the formation of the 1'-sulfoxymetabolite and subsequently formed DNA adducts play an important role in the alkenylbenzene-induced carcinogenicity, and that knowledge of the levels of DNA adduct formation in human livers at relevant dietary intake levels would be of use to facilitate risk assessment for low-dose alkenylbenzenes intake. In this respect it is of interest to note that Herrmann et al. (2013) reported detection of methyleugenol DNA adducts in liver samples obtained from human subjects. Methyleugenol DNA adducts in the livers of these human subjects likely results from dietary intakes. The results of the present study imply that it would be of interest to look for also other alkenylbenzene DNA adducts in human liver samples. It would also be of use to extend the already-developed PBK models in humans to physiologically based dynamic (PBD) models that enable prediction of DNA adduct formation in human liver upon exposure to selected alkenylbenzenes. In a previous study by Paini et al. (2012) such a PBD model for liver DNA adduct formation was developed and validated in an *in vivo* study for estragole DNA adduct formation in the liver of rats. This study also revealed that

not only bioactivated to the reactive 1'-sulfoxymetabolite but also DNA adduct formation in rat liver was linear with the dose of estragole (Paini et al., 2010, 2012). Development of such PBD models for liver DNA adduct formation in human could be achieved by combining the existing human PBK models with data on alkenylbenzene concentration-dependent DNA adduct formation in isolated human hepatocytes as done for rat hepatocytes exposed to estragole previously (Paini et al., 2010, Paini et al., 2012). Such studies in primary hepatocytes are likely to also account for possible repair and stability of the alkenylbenzene adducts and would thus provide insight in whether formation of adducts, even during a short period, could have irreversible effects and thus be deleterious, or whether they are likely to be repaired, thereby decreasing risk. Another issue to consider in this respect is whether it would be realistic that intake is not prolonged over time, especially in individuals who regard PFS as healthy and natural. A cross-sectional, 12 month retrospective survey that has been conducted in 24 cities in six European countries, by the PlantLIBRA consumer survey (2014), revealed that PFS product usage patterns are most often taken 'periodically' (37.3 %), when experiencing a 'worsening of a condition' (22.2 %), 'whenever/sporadically' (19.8 %), and on 'other non-specified occasions' (17.8 %). Periodically meaning 'once daily for at least 2 consecutive or non-consecutive weeks, more doses per week for at least 3 consecutive weeks, more doses per week for at least 4 consecutive or non-consecutive weeks, or 2 or more different PFS, in an appropriate dose form, at a minimum frequency of 1 or more doses per week, with the sum of the usage period of the 2 or more products being equal to at least 4 weeks (Garcia-Alvarez et al., 2014). This result indicates that some parts of the population may tend to use such products indeed for longer periods of time, whereas others may show more restricted periods of use. It is concluded that the

results of the present paper reveal that daily nutmeg-based PFS consumption according to recommendations for daily intake for especially longer periods of time raise a concern.

CONFLICT OF INTEREST

Prof. IMCM Rietjens declares she is a member of the Expert Panel of the Flavour and Extract Manufacturers Association (FEMA). Other authors declare that no conflict of interest exists.

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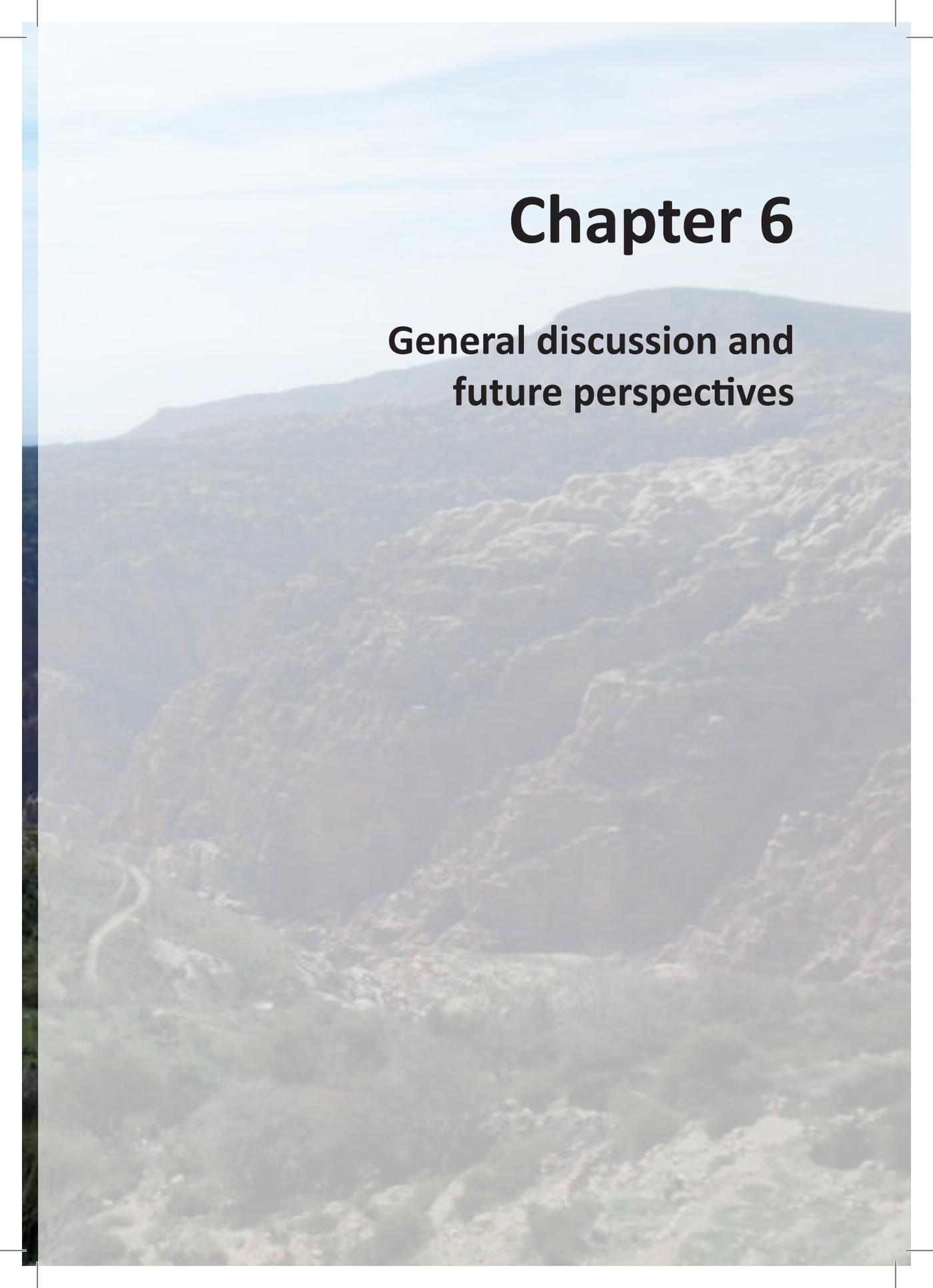
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Chapter 6

**General discussion and
future perspectives**



GENERAL DISCUSSION

The present thesis focusses on the alkenylbenzenes myristicin, apiol, safrole, estragole, elemicin and methyleugenol. These alkenylbenzenes are naturally occurring in a variety of botanicals including anise, star anise, fennel, sweet fennel and parsley, belonging to the plant family *Umbelliferae*, nutmeg and mace, belonging to the plant family *Myristicaceae*, sweet and exotic basil, belonging to the family *Labiatae*, and tarragon, belonging to the plant family *Compositae*. Exposure to myristicin, safrole, estragole, and methyleugenol via food occurs mostly from basil, nutmeg, and parsley. The range of estimated daily intakes (EDIs) of these alkenylbenzenes from the use of spices and spice oils ranges from 0.9 to 166 µg/kg bw per day, with the EDI of safrole being the lowest and that for estragole the highest (JECFA 2009). The EDIs of elemicin and apiol from all sources make a minor contribution to overall intake (Gavin et al. 2007). With respect to alkenylbenzene exposure, pesto paste eaters are considered the highest exposed group, with 20 mg methyleugenol on average in each meal, making the EDI amount to 250 µg/kg bw per day of methyleugenol for a 70 kg person (Miele et al. 2001).

The alkenylbenzenes safrole, myristicin, apiol, estragole, methyleugenol, and elemicin are structurally related, and they may share similar metabolism, mode of action and biological effects (JECFA 2009; van den Berg et al. 2012). Botanicals and botanical preparation containing alkenylbenzenes may be of concern because these compounds may be genotoxic and carcinogenic displaying similar characteristics regarding tumor formation (Phillips et al. 1981; Miller et al. 1983; Randerath et al. 1984; Wiseman et al. 1985; NTP 2000; Zhou et al. 2007; Kobets et al. 2016). For myristicin and apiol, studies that show the ability to induce liver cancer are not available. The similarity of myristicin and apiol to safrole provides the basis for a mode

of action based risk assessment using physiologically based kinetic (PBK) modelling based read-across from safrole for which adequate rodent tumor data are available.

PBK models, in general, are developed in order to study effects related to interspecies differences, intra-species differences, high to low dose extrapolations, route to route extrapolations, and exposure scenario extrapolations. PBK models integrate diverse sets of kinetic data, and can predict tissue dosimetry for situations relevant to human dietary intake of chemicals of interest.

For the assessment of the potential risks of human exposure to compounds that are both genotoxic and carcinogenic, the European Food Safety Authority (EFSA) recommended the Margin of Exposure (MOE) approach to evaluate the priority for risk management actions (EFSA 2005; EFSA 2009a). The MOE compares the exposure levels causing malignant tumors in experimental animals with dietary intake estimates (EDIs) in humans, considering differences in consumption patterns. To obtain the MOE, EFSA recommends the use of the BMDL₁₀ (the lower confidence limit of the benchmark dose resulting in 10% extra cancer incidence) (EFSA 2009b). The BMDL₁₀ is a standardized reference point derived, by mathematical modelling, from the animal data, and falls within the observed range of experimental data (EFSA 2009a). In previous studies, the BMDL₁₀ values for safrole, estragole, and methyleugenol could be derived from *in vivo* tumor data (van den Berg et al. 2011). However, not for all alkenylbenzenes tumor data enabling definition of a BMDL₁₀ for risk assessment are available. Such data are not available for elemicin, myristicin and apiol. Previously, a BMDL₁₀ value for elemicin was derived by read-across from methyleugenol and estragole (van den Berg et al. 2012).

The aim of the present study was to develop a MOA and PBK modeling based approach for read-across in risk assessment from safrole for which *in vivo* toxicity studies are available to the

related compounds myristicin and apiol for which tumor data are not reported, thus contributing to alternatives in animal testing, and to use the outcomes for risk assessment for the consumption of PFS and other botanical products containing myristicin and structurally related alkenylbenzenes considering individual and combined exposure.

In chapter 2 of this thesis, physiologically based kinetic (PBK) models were developed for myristicin by extension of the PBK models for the structurally related alkenylbenzene safrole in rat and human. The newly defined models provide a quantitative insight into the consequences of dose- and species- dependent effects on bioactivation of myristicin. The models were based on *in vitro* metabolic parameters determined using relevant tissue fractions, *in silico* derived partition coefficients, and physiological parameters derived from the literature. The models consist of six compartments including liver as metabolizing compartment, and separate compartments for fat, arterial blood, venous blood, richly perfused, and slowly perfused tissues. Following an exposure to dose levels of 0.05 mg/kg bw to 300 mg/kg bw myristicin, plasma and liver levels of myristicin and its proximate carcinogenic metabolite 1'-hydroxymyristicin were predicted showing myristicin to be almost completely metabolized within a 720-hr period in rat and human. Comparing the predicted metabolite formation at 0.05 and 300 mg/kg bw, the percentage of the dose converted to the proximate carcinogenic metabolite 1'-hydroxymyristicin was predicted to increase in a dose-dependent manner. Comparison of the relative extent of bioactivation of myristicin by rat and human liver revealed that formation of 1'-hydroxymyristicin (expressed as nmol/g liver) is comparable in rat and human liver at the low dose of 0.05 mg/kg bw and 1.8-fold higher in rat liver than in human liver at a dose of 300 mg/kg bw. Formation of the ultimate carcinogenic metabolite 1'-sulfoxymyristicin was predicted to be

4-fold higher in human liver than in rat liver at a low dose of 0.05 mg/kg bw and 2.8-fold higher in human liver than in rat liver at a dose of 300 mg/kg bw.

It was also shown that glucuronidation of 1'-hydroxymyristicin, representing a detoxification pathway, is the most important pathway in rat, while its oxidation is the most important pathway for conversion of 1'-hydroxymyristicin in human. Moreover, on the basis of the kinetic data obtained, bioactivation of 1'-hydroxymyristicin following sulfonation was found to represent only a minor pathway in both rat and human.

The PBK models for myristicin were comparable to the PBK models for estragole, methyleugenol, and safrole, for which more data allowing the evaluation of the models were available. The performance of the rat and human PBK models developed for estragole, methyleugenol, and safrole was reported before (Punt et al. 2008; Punt et al. 2009; Al-Subeihi et al. 2011; Martati et al. 2011; Al-Subeihi et al. 2012; Martati et al. 2012). Evaluation was done by comparing the predicted levels of a variety of metabolites in plasma or excreted in the urine of rats and the predicted blood concentrations in human to comparable data reported in the literature. These comparisons revealed that the predicted PBK model values and the levels of these metabolites derived from *in vivo* studies adequately matched. The performance of the newly developed PBK models for myristicin could not be evaluated against *in vivo* data because quantitative data on the formation or excretion of the different metabolites in rat or humans exposed to this compound are not available. The only data reported that were of use were the data by Beyer et al. (2006) reporting that for both rats and humans exposed to nutmeg, the corresponding *O*-demethylenation metabolite of myristicin was the predominant metabolite, exceeding other metabolites by at least a factor of 10. In rat urine samples collected over a 24-hr period after administration of a single oral dose of 100 mg/kg bw myristicin, urinary metabolites

resulting from *O*-demethylenation amounted to 67 % of the total dose (Beyer et al. 2006). In line with these results, the developed rat PBK model predicted the *O*-demethylenated myristicin metabolite 5-allyl-2,3-dihydroxyanisole to be the major metabolite formed at a dose of 100 mg/kg bw myristicin in rat after 24-hr, with a percentage of 73 % of the total dose. This predicted value of 73 % of the dose matches well with the 67 % observed in the *in vivo* rat study and it was concluded that the developed PBK models for myristicin will also adequately describe the *in vivo* levels of metabolites formed in rat and human after conversion of myristicin and 1'-hydroxymyristicin at different oral dose levels of myristicin. Comparison of the PBK model-based predictions of the bioactivation of myristicin by rat and human to that of its structurally related compound safrole, reveals that the formation of the DNA-reactive 1'-sulfoxy metabolite is comparable; in human formation of the 1'-sulfoxy metabolite is predicted to be 1.35-fold higher for safrole than for myristicin at a low dose level 0.05 mg/kg bw, and 1.1-fold higher for myristicin at high dose level 100 mg/kg bw, in rat formation of the 1'-sulfoxy metabolite is predicted to be 1.5-fold higher for myristicin than for safrole at low dose level 0.05 mg/kg bw, and 2.2-fold higher for myristicin at high dose level 100 mg/kg bw. This shows that the dose-response function for myristicin is steeper (higher curve slope), and that the formation of the 1'-sulfoxymetabolite is higher for safrole at lower doses, but that the order changes to have the formation of the 1'-sulfoxymetabolite of myristicin being higher than that of safrole at higher doses. The PBK model outcomes obtained for the formation of reactive 1'-sulfoxy metabolites of myristicin and safrole can be compared to the relative bioactivation of these two alkenylbenzenes observed in other studies (Phillips et al. 1984; Randerath et al. 1984; Zhou et al. 2007). ³²P-postlabeling indicated that safrole and myristicin have the ability to produce DNA adducts in adult mouse liver upon intraperitoneal injections, amounting to levels of 17.5 and 7.8 pmol/mg

DNA, respectively (Phillips et al. 1984). In another study the covalent binding to adult female mice CD-1 liver of safrole was 2.7 times higher than that of myristicin at low dose (2 mg/mouse) and 4.0 times higher at higher dose (10 mg/mouse) (Randerath et al. 1984). Using human HepG2 cells exposed *in vitro*, the level of DNA adduct formation by safrole was 1.6-fold higher than that for myristicin at low concentration (50 $\mu\text{mol/l}$), while at higher concentration (450 $\mu\text{mol/l}$), the level of adduct formation for myristicin was 1.6-fold higher than for safrole (Zhou et al. 2007).

Altogether, the results obtained support the possibility to perform the risk assessment for myristicin based on the MOE approach using the BMDL_{10} of safrole for tumor formation. Thus, risk assessment of myristicin may be based on the BMDL_{10} derived for safrole of 1.9–5.1 mg/kg bw per day (van den Berg et al. 2011). Using an estimated daily intake of myristicin of 0.0019 mg/kg bw per day (Gavin et al. 2007) resulting from the use of herbs and spices, this results in MOE values for myristicin that amount to 1000–2700, indicating a priority for risk management. In chapter 3, a similar study was undertaken for apiol. To this end PBK models for apiol were developed in order to facilitate risk assessment based on read-across from the related alkenylbenzene safrole. The formation of the different microsomal metabolites of apiol in rat liver by the PBK model-based predictions was dose dependent with a relative increase in bioactivation of apiol at higher doses.

With the model obtained the relative extent of bioactivation and detoxification of apiol at different oral doses was examined. At low doses, formation of 4-allyl-3,6-dimethoxy benzene-1,2-diol (ADD) and 2',3'-dihydroxyapiol (DHA) leading to detoxification appeared to represent the major metabolic pathways, occurring in the liver. At high doses, the rat model revealed a relative increase in the percentage of formation of the proximate carcinogenic metabolite 1'-

hydroxyapiol; from 4% to about 7% of the dose at low and high dose levels respectively, occurring in the liver. By comparing the PBK model based predicted 1'-sulfoxy metabolite formation of apiol with that for safrole, it appeared that at similar dose levels the formation of the 1'-sulfoxy metabolite for apiol is predicted to be about 3-fold lower than for safrole in rat liver and 2-fold lower in human liver. Based on these PBK modeling results, making a correction for the expected 3-fold lower formation of the ultimate carcinogenic 1'-sulfoxy metabolite at similar dose levels, the apiol BMDL₁₀ value would be 3 times the BMDL₁₀ for safrole. Based on an estimated BMDL₁₀ for apiol of 5.7 to 15.3 mg/kg bw per day and an estimated daily intake of 4×10^{-5} mg/kg bw per day, the margin of exposure (MOE) would amount to 140000 to 385000, and this indicates a low priority for risk management.

In Chapter 4, a more detailed analysis of the risks of the presence of alkenylbenzenes in basil-containing sauce of pesto was made using the MOE approach and taking into consideration the possible combined exposure to different alkenylbenzenes. The determination of the levels of alkenylbenzenes in basil-containing sauce of pesto revealed that all the samples analysed contained methyleugenol. In addition to methyleugenol, some samples contained estragole, myristicin, and/or apiol. Based on chemical analysis the EDI values could be determined assuming a body weight of 70 kg and intake of 10 g of basil (based on wet weight) per meal (Miele et al. 2001), provided by an amount of pesto sauce that could be calculated taking into account the basil content (%) in the pesto sauce as indicated on the label. For samples containing only methyleugenol, the EDI values for methyleugenol ranged from 3.2–44.3 µg/kg bw per day. These EDI values resulted in MOE values of 345–4781. Since several alkenylbenzenes were found in some of the pesto samples, and these alkenylbenzenes show high similarity in structure, target organ, type of adverse effects and mode of action through formation of a DNA reactive 1'-

sulfoxymetabolite contributing to formation of liver tumors, a combined exposure assessment and subsequent risk assessment were also performed. For this combined risk assessment, the EDIs were calculated assuming equal potency of the alkenylbenzenes and also based on use of the so-called Toxic Equivalency (TEQ) approach. The Toxic Equivalency Factors (TEFs) for the different alkenylbenzenes were defined using methyleugenol as the reference compound (TEF=1). The TEF values for the other compounds were defined using different sets of literature data, including data for *in vivo* DNA adduct formation in the liver (Randerath et al. 1984) and also the PBK model based predictions for formation of the ultimate carcinogenic 1'-sulfoxy metabolites and the BMDL₁₀ values obtained by read-across from safrole as defined for myristicin and apiol in Chapter 2 and Chapter 3 of the thesis, as well as the previously derived BMDL₁₀ values for methyleugenol and safrole (van den Berg et al. 2011). In samples containing multiple alkenylbenzenes, the MOE values resulting from combined exposure assessment were 1.5–5-fold lower than the MOE values obtained when considering the presence of only methyleugenol, and are all below 10000. Combined risk assessment based on equal potency did not vary much from results obtained using the TEQ approach. This can be ascribed to the minor variation in potencies of the different alkenylbenzenes detected in the pesto samples.

It is of interest to note that these MOE values refer to regular daily consumption of pesto which may be an overestimation of realistic human consumption. Levels of methyleugenol in pesto sauce samples and consumption frequency of pesto sauce directly impact the EDI and the resulting MOE values. There is no generally established method for taking a shorter than life time exposure into account but one could consider using Haber's rule. Assuming that Haber's rule would apply, the toxic effect would vary linearly with the time of exposure and the concentration (i.e. $C \times T = k$, where C is concentration or dose, T is time of exposure, and k is a

constant toxic response for the specific substance) (Doull and Rozman 2000). In Chapter 4 it was evaluated, using Haber's rule, what level of alkenylbenzenes, what amount of basil-based pesto sauce consumed and/or what exposure frequency would result in an MOE of 10000 or higher and be of low priority for risk management. Based on that analysis it was concluded that consumption of pesto sauces would especially be of concern if consumed on a daily basis over longer periods of time. The results of chapter 4 reveal that pesto consumption does not always represent a cancer risk and provide insight into when this might be the case and under which level and frequency of consumption pesto consumption would not raise a concern.

In Chapter 5, a risk assessment of nutmeg-based plant food supplements (PFS) containing different alkenylbenzenes was performed based on the alkenylbenzene levels quantified in a series of PFS collected via the online market. Based on the alkenylbenzene contents detected and the use levels of the respective PFS recommended on the labels, EDI values calculated for individual alkenylbenzenes ranged from 0.3 to 312 $\mu\text{g}/\text{kg}$ bw per day. Using the respective BMDL_{10} values these EDI values resulted in MOE values that were generally below 10000 ranging from 6 to 7870. A combined exposure assessment and subsequent risk assessment were performed for those PFS in which more than one alkenylbenzene was detected. Assuming equal potency of the alkenylbenzenes, combined EDI values obtained ranged from 1.5 to 631 $\mu\text{g}/\text{kg}$ bw per day resulting in MOE values ranging from 3 to 1309 for the samples in which more than one alkenylbenzene was detected. Using a TEQ concept and the Toxic Equivalency Factors (TEFs) for the different alkenylbenzenes defined as described in Abdul et al. (2016) using safrole as the reference compound (TEF=1) (Alajlouni et al. 2016b). Combined EDI values ranged from 0.4 to 295 μg safrole equivalents/kg bw leading to MOE values ranging from 6 to 5020. MOE values resulting from both these combined exposure assessments were up to 5-fold lower than the MOE

values obtained when considering individual exposure to alkenylbenzenes. The MOE values obtained for the combined risk assessment based on the TEQ concept were higher than the ones obtained when assuming equal potency. This can be ascribed to the fact that the TEF values of the alkenylbenzenes present in the PFS, including myristicin, elemicin, and methyleugenol, are lower than that of safrole leading to lower EDI values when expressed in safrole equivalents and higher MOE values.

It is of interest to note that these MOE values refer to regular daily consumption of nutmeg-based PFS during a whole lifetime which may be an overestimation of realistic human consumption. Levels of alkenylbenzenes in nutmeg-based PFS samples and consumption frequency directly impact the EDI and the resulting MOE values. Using Haber's rule to correct for shorter than life time exposure it was shown that limiting exposure to only one or two weeks could result in MOE values that would be, with the levels of alkenylbenzenes detected in the PFS in the study and the proposed uses of these PFS, higher than 10000 and thus of low priority for risk management. It is concluded that the results of the study reveal that daily nutmeg-based PFS consumption following recommendations for daily intake for especially longer periods of time raises a concern.

FUTURE PERSPECTIVES

This thesis presents the use of physiologically based kinetic (PBK) modeling to facilitate read-across in risk assessment from a compound for which *in vivo* toxicity studies are available to a related compound for which tumor data are not reported, and risk assessment of individual and combined exposure to alkenylbenzenes present in basil-containing pesto and nutmeg-based plant food supplements (PFS). In the next sections some suggestions are given for steps to be taken in the near future for further improvement of risk and safety assessment of individual and combined exposure to alkenylbenzenes from botanical preparations. Such steps include (1) Further development of the PBK models and the read-across approach to predict toxicological reference points for risk assessment of genotoxic carcinogens, (2) incorporation of the dynamic characteristics of the active chemical substance(s) in the *in silico* models, (3) additional considerations for the MOE based risk assessment including the incorporation of Chemical Specific Adjustment Factors (CSAFs) for interspecies and interindividual variation in kinetics within the human population, (4) long to short-term exposure adjustment, (5) further improvement of the TEF approach, (6) banding of the MOE values and related conclusions for risk management, and (7) quality assurance of food supplements and botanical preparations.

Further development of the PBK models and the read-across approach to predict toxicological reference points for risk assessment of genotoxic carcinogens

The MOE approach needs carcinogenicity data to define the BMDL₁₀. However, not for all alkenylbenzenes tumor data enabling definition of a BMDL₁₀ for risk assessment are available. Such data are missing for myristicin and apiol. So, a mode of action based PBK model approach was developed in chapter 2 and chapter 3 to perform a read-across from a compound for which

rodent tumor data are available (safrole) to these compounds for which such data are not available (myristicin and apiol). The read-across was based on the similarity in structure, metabolism, mechanism of toxicity, and ultimate adverse effect of the alkenylbenzenes. Previously, a BMDL₁₀ value for elemicin was derived by PBK model based read-across from methyleugenol and estragole (Van den Berg et al. 2012). The PBK models quantify the dose-dependent formation of the ultimate carcinogenic 1'-sulfoxymetabolite thereby facilitating the read-across based on predictions of relative bioactivation. It is important to realise that the PBK models as developed for the read-across in the present thesis required a large number of parameters many of which needed to be defined for each individual compound. This makes the development of these models time and effort consuming. For that reason, for efficient future development of this PBK model based read-across approach priority should be given to the development of generic PBK models for groups of related compounds to facilitate an efficient and time-and-cost-saving risk assessment on genotoxic carcinogens for which rodent tumor data are not available (Bessemers et al. 2014). The models developed for the alkenylbenzenes, and especially the results of the sensitivity analyses, could be of use since they define and evaluate the influence of the various input parameters on the output. This could be of great use in the development of generic models for other botanical-borne naturally occurring group of compounds, focusing on those aspects in the model that affect the predictions most. Another category of botanical ingredients of concern for which limited tumor data for their risk assessment are available and for which PBK model based read-across would facilitate risk assessment are the pyrrolizidine alkaloids (Chen et al. 2017).

Some efforts in development of generic PBK models have already been reported. Brightman et al. (2007) introduced a generic model PBK model that was parameterized for human physiology

predicting the *in vivo* pharmacokinetics of compounds for which plasma levels in human have been determined following an intravenous dose, concluding that the generic PBK model can be a powerful, efficient, and cost-effective tool for toxicokinetic predictions and reduction of *in vivo* animal experimentation. Chemical-specific data required by the model are generated from *in silico* (QSAR) and/or *in vitro* procedures. For oral exposure; generic PBK models likely will require quantitative values for intestinal barrier permeability, a measure of lipophilicity, an estimate of the fraction of the compound bound to plasma proteins, and an estimate of intrinsic hepatic clearance (Brightman et al. 2005; Brightman et al. 2006; d'Yvoire et al. 2007). Depending on the group of chemicals under study a few compound specific parameters may have to be added but the PBK model development could start from the generic basic model and its evaluation before adding additional parameters. Focusing on a limited set of parameters and definition of validated generic models will facilitate future applications of the PBK model based read-across presented in the present thesis for the alkenylbenzenes.

Incorporation of the dynamic characteristics of the active chemical substance(s) in the *in silico* models

For refinement of the read-across process in risk assessment of genotoxic carcinogens, dynamic processes including DNA-adduct formation, DNA repair, mutation modulation, gene and protein expression and tumor formation occurring at low realistic intake levels should be considered in addition to already predicted kinetics (bioactivation and detoxification) of myristicin and apiol (Chapter 2 and 3). The dynamic process could be described using Physiologically Based Dynamic (PBD) models.

Extending the PBK models to model the possible levels of DNA adduct formation upon exposure to the alkenylbenzenes has been done before for estragole (Paini et al. 2010). To this end, the PBK model describing the formation of the proximate carcinogenic 1'-hydroxymetabolite, was extended by linking the area under the curve for 1'-hydroxyestragole formation predicted by the PBK model to the area under the curve for 1'-hydroxyestragole in the *in vitro* experiments on DNA adduct formation in rat primary hepatocytes exposed to 1'-hydroxyestragole.

In this model formation of 1'-sulfoxyestragole as well as DNA repair were implicitly included, since these reactions occur in the isolated hepatocytes and thus affect the ultimate levels of DNA adduct formation detected. The PBD model for liver DNA adduct formation thus developed for rats predicted the formation of E-3'-N²-dGuo in the liver of rat at 400 mg/ kg bw per day, quantified by LC-ESI-MS/MS, at a level amounting to 4 adducts in 10000 nucleotides. This value was within the same order of magnitude as DNA adduct formation in the liver of mice exposed to estragole determined using ³²P-postlabeling detecting 1 adduct in 10000 nucleotides (Randerath et al. 1984).

The results obtained revealed that DNA adduct formation was adequately predicted and also that not only bioactivation to the reactive 1'-sulfoxymetabolite but also DNA adduct formation in rat liver was linear with the dose of estragole (Paini et al. 2010). Such studies also account for possible repair and stability of the alkenylbenzene adducts and would thus provide insight in whether formation of adducts, even during a short period, could have irreversible effects and thus be deleterious, or whether they are likely to be efficiently repaired, thereby decreasing risk. Also prediction of DNA adduct formation is one step closer to the ultimate mutagenicity and carcinogenicity than the predicted 1'-sulfoxyestragole formation, thus providing an even better basis for read-across.

Additional considerations for the MOE based risk assessment including the incorporation of Chemical Specific Adjustment Factors (CSAFs) for interspecies and interindividual variation in kinetics within the human population

Many qualitative and quantitative approaches can be used to assess the risk of genotoxic carcinogenic compounds. Data used in these approaches are mainly obtained from rodent bioassays performed at high dose levels. This implies that extrapolation to lower exposure levels is required in order to judge the risks at realistic human daily exposure levels. Such an extrapolation may be far outside the experimental data range and up to five orders of magnitude below the observable response in the animal bioassay. EFSA questioned if such an extrapolation is justified, and denies quantitative risk assessment based on high to low dose extrapolation as a scientifically acceptable approach (Barlow et al. 2006; O'Brien et al. 2006; Benford et al. 2010). EFSA considered, amongst other reasons, that this extrapolation depends too much on the model applied, that evidence for the frequently used linear extrapolation model is not available, and that this extrapolation of rodent tumor data to human risk levels does not take into account species differences. As an alternative EFSA proposed the Margin of Exposure (MOE), defined as the ratio between the BMDL₁₀ and the estimated daily intake (EDI), that was also applied in the present thesis. The EFSA Scientific Committee considered that an MOE of 10000 or more, based on animal cancer bioassay data, *“would be of low concern from a public health point of view and might reasonably be considered as a low priority for risk management actions”* (EFSA 2005). The value of 10000 was considered to include all possible uncertainties that may be involved and affect the risk assessment. It includes a factor of 100 for kinetic and dynamic differences between species and within humans, a factor of 10 that presents the variability in cell cycle control and DNA repair within humans, and a factor of 10 that takes into account that the

BMDL₁₀ represents a 10% BMR (EFSA 2005; O'Brien et al. 2006). Subdivision of the interspecies uncertainty factor of 10 into toxicokinetics and toxicodynamic components would allow part of the default value to be replaced by chemical-specific data consistent with the weight of evidence for mode of action when they are available. The proposed default value of 10 for interspecies differences for example can be considered to consist of a factor of 4 and 2.5 for the toxicokinetics and toxicodynamic components, respectively. This implies that when relevant chemical-specific toxicokinetics and toxicodynamic data on species differences would be available, the default values could be replaced with CSAFs for interspecies differences in toxicokinetics and toxicodynamic (HealthCanada 1994; Renwick 1999; Gundert-Remy and Sonich-Mullin 2002; Meek et al. 2003; IPCS 2005). The default uncertainty factor for interspecies differences in toxicokinetics can be informed through the use of PBK models (Rowland 1985; Andersen et al. 1995; Jarabek 1995). The PBK model based predictions for the formation of the ultimate carcinogenic 1'-sulfoxymetabolite for myristicin and apiol in rat and human defined in the present thesis showed that at low dose in human the formation of the ultimate carcinogenic metabolite is 4 times higher than in rat, while for apiol the difference was 15-fold. These results of the present thesis suggest that a factor of 4 to take into account species differences in toxicokinetics may be sufficient for myristicin but not for apiol. For apiol, the default MOE value of 10000 may increase accordingly which would result in more samples to result in MOE values that might be of concern.

In future, Monte Carlo simulation on the variability in the formation of the 1'-sulfoxymetabolite can potentially be used to derive the CSAFs, which can be used to evaluate the appropriateness of the default factor of 3.16 generally assumed to reflect interindividual variation in kinetics within the human population (Barton et al. 1996; Dourson et al. 1996; Clewell et al. 1999; Price

et al. 1999; Haber et al. 2002; Lipscomb and Ohanian 2007). The results of this thesis illustrate how these PBK models may assist the development of CASFs in risk assessment.

Long to short-term exposure adjustment

The risk assessments presented in this thesis were based on the MOE approach which defines an MOE based on BMDL₁₀ values from chronic carcinogenicity data as well as EDI values assuming daily exposure over a life time. Use of the PFS and of sauce of pesto however, may be less than daily and not over a whole life time. This raises the question how to accommodate these different exposure scenario's in the MOE based risk assessment. One way to extrapolate from longer durations to shorter durations of exposure is through the use of Haber's Law: $c \times t = k$, where c is the dose, t the exposure time and k a constant. This implies a linear relationship between the dose and the exposure time. The use of linear time-to-dose extrapolation to assess the risk from less-than-life-time exposure to genotoxic substances has been discussed by various authors, i.e. if linearity is assumed, then it is important to determine if Haber's Rule ($C \times T$) is appropriate for the exposure scenario of concern, Haber's rule supports distributing the lifetime dose over the total number of exposure days during less-than-lifetime exposure, thereby allowing for a higher daily intake than would be the case for lifetime exposure. If the response is nonlinear, it should be critically evaluated whether application of Haber's law is appropriate in estimating a toxicological reference point, taking into account the MOA and TK/TD based considerations. If Haber's law applies, several approaches could be considered, i.e. using Haber's law strictly without adjustment, adjusted by application of a Dose-Response Correction Factor (DRCF), the magnitude of which could vary according to the exposure scenario and/or what is known about the MOA, or according to predefined exposure scenarios (acute for 14 days

or fewer, short-term ≥ 14 days to 1 year, intermediate ≥ 1 year to 7 years, chronic ≥ 7 years to 70 years), and assign “default” adjustment factors (Bos et al. 2004; Callis et al. 2010; Felter et al. 2011).

Felter et al. (2011) indicated that it is important to acknowledge the assumptions underlying this methodology of linear time-to-dose extrapolation, which include the fact that chemical-specific carcinogenicity data should be available and that the data support a linear dose-response relationship (Felter et al. 2011). Whether such a linear dose-response relationship would apply to the dose dependent induction of liver tumors by the alkenylbenzenes remains to be established and may depend on the MOA underlying the carcinogenicity. This mode of action proceeds by cytochrome P450-catalyzed hydroxylation to a 1'-hydroxymetabolite, followed by sulfotransferase (SULT)-mediated conversion to a 1'-sulfoxymetabolite (Phillips et al. 1981). This metabolite is unstable and reacts with DNA. Studies using PBK modelling revealed that the bioactivation of alkenylbenzenes to their ultimate carcinogenic 1'-sulfoxy metabolites is linear with the dose, from dose levels as low as levels of realistic human dietary intake up to dose levels as high as the BMDL₁₀ and BMD₁₀ inducing liver tumors in rodent bioassays (Punt et al. 2009; Rietjens et al. 2010; Al-Subeihi et al. 2012; Martati et al. 2012). The mutagenic potential of the 1'-sulfoxy alkenylbenzene DNA adducts has not been extensively quantified so far. Herrmann et al. (2012) investigated the mutagenic potential of sulfate conjugates of 1'-hydroxymethyleugenol and showed that different isomeric hydroxylated metabolites of methyleugenol are mutagenic in an Ames test using *Salmonella typhimurium* TA100 strains expressing different sulfotransferase enzymes (Herrmann et al. 2012). This suggests that the 1'-sulfoxymetabolite and the subsequently formed DNA adducts play an important role in the alkenylbenzene-induced carcinogenicity, and that knowledge of the levels of DNA adduct

formation in human livers at relevant dietary intake levels would be of use to facilitate risk assessment for low-dose alkenylbenzenes intake. In this respect it is of interest to note that Herrmann et al. (2013) reported detection of methyleugenol DNA adducts in liver samples obtained from human subjects (Herrmann et al. 2013). Methyleugenol DNA adducts in the livers of these human subjects likely results from dietary intakes. The results of the present study imply that it would be of interest to look for also other alkenylbenzene DNA adducts in human liver samples. It would also be of use to extend the already-developed PBK models in humans to physiologically based dynamic (PBD) models that enable prediction of DNA adduct formation in human liver upon exposure to selected alkenylbenzenes. As already outline above in a previous study by Paini et al. (2012) such a PBD model for liver DNA adduct formation was developed and validated in an *in vivo* study for estragole DNA adduct formation in the liver of rats. This study also revealed that not only bioactivation to the reactive 1'-sulfoxymetabolite but also DNA adduct formation in rat liver was linear with the dose of estragole (Paini et al. 2010; Paini et al. 2012). Development of such PBD models for liver DNA adduct formation in human could be achieved by combining the existing human PBK models with data on 1'-hydroxyalkenylbenzene concentration-dependent DNA adduct formation in isolated human hepatocytes as done for rat hepatocytes exposed to 1'-hydroxyestragole previously (Paini et al. 2010; Paini et al. 2012). Thus the available data on bioactivation and DNA adduct formation of alkenylbenzenes show linearity and thereby provide kinetic and some dynamic data to support use of Haber's rule.

Another issue to consider when evaluating short term exposure scenarios is whether it would be realistic that intake is not prolonged over time, especially in individuals who regard PFS as healthy and natural. A cross-sectional, 12-month retrospective survey that has been conducted in 24 cities in six European countries, by the PlantLIBRA consumer survey (2014), revealed that

PFS product usage patterns are most often taken “periodically” (37.3%), when experiencing a “worsening of a condition” (22.2%), “whenever/sporadically” (19.8%), and on “other non-specified occasions” (17.8%). Periodically meaning “once daily for at least 2 consecutive or non-consecutive weeks, more doses per week for at least 3 consecutive weeks, more doses per week for at least 4 consecutive or non-consecutive weeks, or 2 or more different PFS, in an appropriate dose form, at a minimum frequency of 1 or more doses per week, with the sum of the usage period of the 2 or more products being equal to at least 4 weeks” (Garcia-Alvarez et al. 2014). This result indicates that some parts of the population may tend to use such products indeed for longer periods of time, while others may show more restricted periods of use. The results of the present thesis illustrated how, using Haber’s rule, the risks of different exposure scenario’s, including scenario’s with restricted periods of intake, could be evaluated. The results of the present thesis reveal that based on the MOE approach without a correction from long to short term exposure, daily consumption of PFS following recommendations for daily intake raises a concern.

Further improvement of the TEF approach

When analysing alkenylbenzene containing foods including the sauce of pesto and nutmeg-based PFS it appeared that many preparations contain more than one alkenylbenene, pointing at combined exposure (Alajlouni et al. 2016b; Al-Malahmeh et al. 2017a). This was also reported before by other authors (Meek et al. 2011; EFSA 2013; Pose-Juan et al. 2016; Alajlouni et al. 2016b; Al-Malahmeh et al. 2017b). For this reason risk assessment of combined exposure to alkenylbenzenes was performed for nutmeg-based PFS and basil-based pesto sauce.

For alkenylbenzenes, known to have similar chemical structures, metabolic pathways and a similar MOA underlying induction of a similar adverse effect, and for which it can be assumed that they will display similarly shaped dose-response curves, the combined toxicity of the individual components can be estimated by so-called dose-addition using the sum of their doses, equally or scaled for potency relative to that of another component of the mixture for which adequate dose-response information is available. Based on the dose addition concept, the TEF methodology applies. Application of this methodology in human health risk assessment has been described and reaffirmed for use by for example the guidance of the Environmental Protection Agency (EPA) entitled: “*Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures*” (EPA 2000). In practice, the scaling factor for each chemical in the group is typically based on a comparison of its toxic potency to that of a designated index chemical. The index chemical is well-studied toxicologically and must have a dose-response function to apply the methodology to a mixture. Toxicological data considered for these comparisons of toxic potency can be derived from both *in vitro* and *in vivo* studies as well as from structure-activity relationships. The relative potencies can also be defined based on biochemical changes, toxicity, and/or carcinogenicity.

Application of the TEF approach to the human health risk assessment of chemicals carries with it some uncertainties, i.e. the assumption of parallel dose-response curves, which is difficult to show particularly in the low response region of interest, and the assumption that the estimate of relative potency (REP) from animal studies are predictive of REPs in humans. In the process and data used to derive TEF (or REP) values, even more uncertainties evolve i.e. variability in the kind of information available in many types of *in vivo* and *in vitro* studies for comparing the responses. Safrole and methyleugenol were both used as index compound in this thesis,

depending on their presence in the mixture of the botanical preparation studied. Both, safrole and methyleugenol are well-studied toxicologically and have a well-defined dose-response function for various endpoints on which the TEF values could be based including data from carcinogenicity studies, data on *in vivo* DNA adduct formation and data on formation of the reactive 1'-sulfoxy meytabolites.

A number of different methods are employed to calculate REP (or TEF) values (Haws et al. 2006), with the uncertainty in Toxic Equivalence (TEQ) estimates and in the TEF methodology being highly dependent on the exposure estimates used in the TEQ calculations. The BMD approach is suitable for the definition of TEF as it enables the estimation of equipotent doses by interpolation between applied doses, and it has been used to provide relative potency estimates for different organophosphates (Bosgra et al. 2009), for estimating equipotent doses in *in vivo* and *in vitro* genotoxicity tests (Bemis et al. 2015; Wills et al. 2015; Soeteman-Hernandez et al. 2015a; Soeteman-Hernandez et al. 2015b), and for testing if dose addition applies in chemical mixtures (Kienhuis et al. 2015). In this thesis combined risk assessment, based on either equal potencies and also based on relative potency of the different alkenylbenzenes was considered, the latter using TEF values. In an ideal situation such TEF values should be based on the endpoint under consideration, i.e. tumor formation. The carcinogenic potency of alkenylbenzenes was shown in previous studies (Miller et al. 1983; Phillips et al. 1984; Randerath et al. 1984; Zhou et al. 2007), but these studies focused on only a few of the alkenylbenzenes under study and did not provide data for myristicin and apiol that could be compared to those of safrole, estragole and myristicin to set TEF values. Therefore in the present thesis TEF values were determined using BMDL₁₀ values obtained via read-across from safrole, but also surrogate endpoints including relative formation of the ultimate carcinogenic 1'-sulfoxy metabolite and DNA adduct formation

upon *in vivo* exposure. The MOE values obtained for the combined risk assessment based on the TEQ concept did not vary much from the ones obtained when assuming equal potency. This can be ascribed to the fact that the TEF values of the alkenylbenzenes present in botanical preparations analysed vary by less than 2-fold. Therefore it can be concluded that for a first Tier risk assessment on combined exposure to mixtures of alkenylbenzenes dose addition without correction of differences in relative potencies is adequate and that only in cases where the outcome indicates the need for a refined estimate, i.e. when MOE values are around the threshold value of 10000, a TEF based approach might be needed.

Banding of the MOE values and related conclusions for risk management

MOE values obtained in the present thesis showed wide variation with values above and values below 10000 and some values even being lower than 1000. Even though the MOE approach was introduced to avoid the misleading use of apparently precise risk numbers, the threshold value of 10000 proposed to be used in discriminating whether there is a concern or not introduces a sharp cutoff value. This implies that an MOE of 10, or one of 9000, values that are both < 10000 are both of concern, and one of 11000 or 100000 (both > 10000) are both of no concern, although they may indicate different levels of concern. When using an MOE approach based on the BMDL₁₀ from an animal study, some banding systems have been proposed to aid risk communication. The Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC) has proposed a banding system (i.e. <10000, may be a concern; 10000-1000000, unlikely to be a concern; and >1000000, highly unlikely to be a concern). This suggests some banding of the MOE, but it does not yet proposes how to interpret different values <10000.

One could consider further banding of MOE cut-off values that are < 10000 as follows: MOE < 1000 : Very high concern: MOE = 1000-5000: High concern : MOE = 5000 – 10000: Moderate concern.

Banding was also proposed for the so-called exposure potency index (EPI) approach, an alternative to the MOE approach proposed by Health Canada, in their Human Health Risk Assessment for Priority Substances under the Canadian Environmental Protection Act (HealthCanada 1994). The EPI is calculated as the average exposure in the population divided by the dose in experimental animals that produces a 5% incidence of tumors. The EPI can be regarded as a reciprocal of the MOE and the reciprocals of the EPI values correspond to MOE values of < 5000 (high priority), 5000–500000 (moderate priority) and > 500000 (low priority) (HealthCanada 1994). The biological basis and derivation of the different banding systems were not explained.

In addition to banding the value of the MOE outcome, one could also consider a new approach for banding based on the frequency of consumption, the amount consumed, and/or the content of the chemical of interest. In such a system the considerations discussed above for time-dose extrapolations could be taken into account, to correct the critical MOE value used to make the risk management decision for deviating time or dose regimens.

All together it is concluded that an internationally scientific framework to assess and refine the MOE approach, taking into account shorter than life time exposure, and models for banding of MOE values would be of use, and would improve the potential of the MOE approach for risk assessment.

Quality assurance of food supplements and botanical preparations

Results of the present thesis revealed that food supplements and botanical preparations containing alkenylbenzenes at levels that do raise a safety concern are still freely available on the Dutch market and via the internet. This raises the issue of quality control. The guidelines for *Good Agricultural and Wild Collection Practice of Medicinal and Aromatic (Culinary) plants* are suggested to be applied to the growing and primary processing practices of plants and their derivatives traded and used in the European Union. Hence they apply to the production of all plant materials utilized either in a direct or processed form. They also apply to all methods of production including organic production in accordance with the European regulations. Plant raw material should meet the demands of the consumer and the standards of the highest quality. The presence and concentration of physiologically active substances in a botanical species can vary considerably depending on growth, development and chemical profile, influenced by a number of external factors such as soil quality and water properties, temperature, sunlight, the season of cultivation and time of harvesting. It is equally important that the batches of the botanical raw material undergo appropriate testing before acceptance for further processing to ensure that the concentrations of substances with known physiological effects fall within predetermined limits and allow for the natural variability of the botanical material. Accurate identification of all botanical source material selected for further processing is crucial as well. Care should be taken with the identification to avoid renaming or reclassification or switching of varieties. All participants of the production process, primary producers to traders, are required to keep the traceability of the production process. The most important information about the batch should always follow the material by a batch documentation, and Integrity of botanical material (EUROPAM 2006; FoodSupplementsEurope 2016).

Establishment, applying and assuring a good quality system during botanical product preparation, and practicing in-house, internationally harmonized, and/or transferred valid analytical methods to characterize and to identify the bioactive compounds within these products will facilitate risk assessment and help to guarantee the quality of the final product. Integrated plans and continuous improvement through auditing, correcting and preventing non-conformities should be applied from farming to marketing.

CONCLUSION

This thesis assessed the risks of nutmeg-based PFS and basil-containing pesto sauce products that contain alkenylbenzenes using read-across through building of PBK models for two alkenylbenzenes (myristicin and apiol). This facilitated the estimation of a BMDL₁₀ for myristicin and apiol without the need for a rodent bioassay. These BMDL₁₀ values and the ones derived for structurally related alkenylbenzenes directly from bioassays or via read-across were subsequently used in the risk assessment of exposure to these alkenylbenzenes via nutmeg-based PFS and basil-containing pesto sauce. The overall conclusion of the risk assessment of consumption of basil-based pesto sauce and nutmeg-based PFS revealed that daily lifetime consumption of most of these products would be a priority for risk management. On the other hand, exposure to those products for shorter periods of time will increase the MOE values to levels > 10000 indicating a low priority for risk management. Thus, it can be concluded that the risks associated with the use of these products would be limited, provided intake is for only a few weeks during a lifetime. The thesis also provided new proofs of principle of how PBK model based approaches can facilitate novel non-animal based strategies in risk assessment contributing to the development of alternatives for animal testing.

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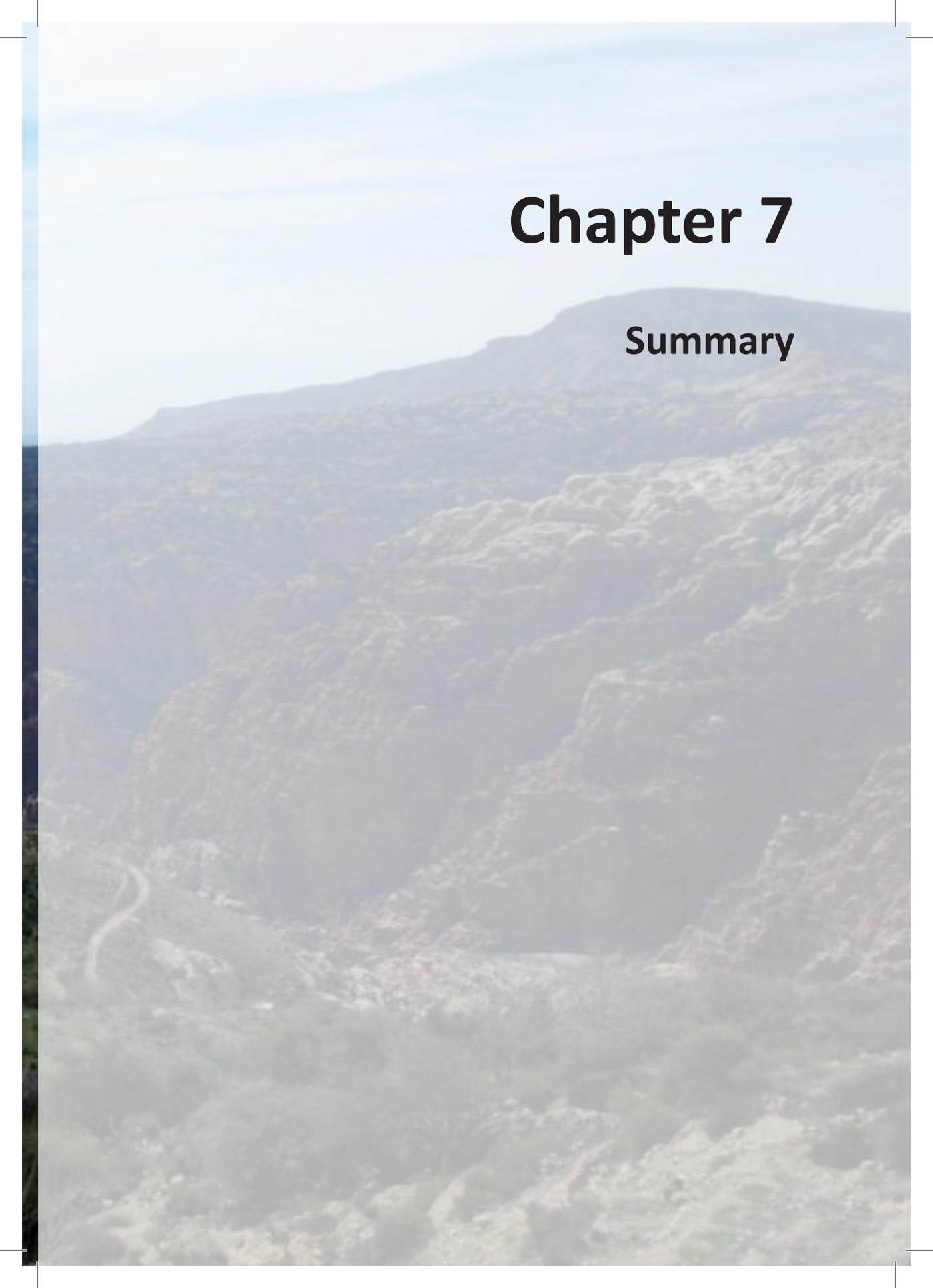
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Chapter 7

Summary



Botanicals and botanical preparations have become widely available on the market in the form of food supplements and other food preparations. In Europe the safety of botanicals and derived food products placed on the market has to comply with the general requirements set out in regulation (EC) No 178/2002. In spite of this regulation, the risks of botanicals and botanical preparations are generally not assessed before they enter the market.

The present thesis aimed to perform a risk assessment of some selected botanicals and derived preparations focusing on samples containing the so-called alkenylbenzenes, including myristicin, apiol, safrole, methyleugenol, elemicin, and estragole. Samples containing alkenylbenzenes may be of concern because these compounds may be genotoxic and carcinogenic displaying similar characteristics regarding mode of action (MOA) and tumor formation. The aim of the present study was to develop a MOA based approach for read-across in risk assessment from safrole, for which *in vivo* toxicity studies are available to myristicin and apiol for which tumor data are not reported, thus contributing to alternatives in animal testing. This was done using physiologically based kinetic (PBK) modelling. Botanical preparations included in the risk assessments performed in the present thesis were basil-containing pesto and nutmeg-based plant food supplements (PFS). Given that several of the preparations analyzed contained several alkenylbenzenes a risk assessment of combined exposure to these alkenylbenzenes was included as well, to give a better understanding of when risk management actions would be needed for botanicals and derived preparations containing these ingredients.

The introduction chapter starts with a short background and definition of the aim of the thesis, followed by a description of the physicochemical properties, natural occurrence and estimated daily intake (EDI) of the alkenylbenzenes, as well as of their ADME (absorption, distribution, metabolism and excretion) characteristics, carcinogenicity and MOA, the relevant risk assessment approach, and finally PBK modelling.

Chapter 2 and 3 of the thesis describe the development of PBK models for myristicin and apiol in male rat and human, enabling the prediction of dose-dependent effects in bioactivation and detoxification of these alkenylbenzenes. This allows comparison of the PBK model-based prediction of bioactivation of myristicin or apiol to the PBK model-based predictions for bioactivation of the structurally related compound safrole, enabling estimation of a BMDL₁₀ for myristicin and apiol from read-across to the BMDL₁₀ available for safrole, thereby facilitating risk assessment of current dietary exposure to myristicin or apiol. The results from PBK analysis support that risk assessment of myristicin may be based on the BMDL₁₀ derived for safrole, and that risk assessment of apiol may be performed using a BMDL₁₀ value of 3 times the BMDL₁₀ for safrole. These results enabled a preliminary risk assessment of current exposure to these alkenylbenzenes showing that the current exposure to myristicin results in MOE values indicating a priority for risk management (Chapter 2), while current exposure to apiol does not raise a concern (Chapter 3). The results obtained illustrate that PBK modeling can facilitate a read-across in risk assessment from a compound for which *in vivo* toxicity studies are available to a related compound for which tumor data are not reported, thus contributing to alternatives in animal testing.

Subsequently, in Chapter 4 the BMDL₁₀ values obtained were used to perform a risk assessment for alkenylbenzenes in basil-based pesto sauce. To this end the levels of methyleugenol and other alkenylbenzenes in basil-containing sauce of pesto were quantified enabling an associated risk assessment based on the MOE approach, taking into consideration the possible combined exposure to different alkenylbenzenes and the BMDL₁₀ values as defined in literature and in chapter 2 and 3 of the thesis. The MOE values obtained generally indicated a priority for risk management when assuming daily consumption of basil-based pesto sauce. It was concluded that

consumption of pesto sauces would be of concern if consumed on a daily basis over longer periods of time.

Chapter 5 evaluates the risk of exposure to myristicin and related alkenylbenzenes through human exposure to nutmeg-based PFS based on the MOE approach. Chemical analysis of various PFS samples was performed and the amount of alkenylbenzenes that would be consumed through consumption of these nutmeg-based PFS were quantified. MOE values were calculated for the individual alkenylbenzenes as well as taking into account the presence of more than one alkenylbenzenes within selected PFS samples. It was concluded that the results reveal that daily nutmeg-based PFS consumption following recommendations for daily intake for especially longer periods of time would raise a concern.

Chapter 6 summarizes the results obtained in the thesis, compiles the overall discussion and presents the future perspectives that follow from the results obtained.



Appendices

Acknowledgements

Curriculum vitae

List of publications

Overview of training activities

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Curriculum Vitae

Amer Al-Malahmeh was born, raised, and grown up in Amman-Jordan. He has graduated from the Chemistry department at the University of Jordan holding a Bachelor Degree in Chemistry, he has been working in different industries, i.e. in utilities maintenance services, carbonated beverages, industrial research, Feed and Fertilizer industry, and Food and Environment Health, Safety, and Quality Laboratories. Meanwhile, he has attended many seminars, training courses, and Associate programs organized locally in Jordan, regionally, and internationally. Those building capacities events were mostly related to quality assurance and technical-oriented issues. 2011, he made a step up to a higher education in the field of measurement Science in Chemistry and earned his Euro-Master degree 2013 from the University of Tartu-Estonia. Afterward, he worked, as a freelancer, with Jordanian accreditation system and European Commission Joint Research Center (IRMM) as a technical Expert and Trainer on Chemistry Laboratories Fit-For-Purpose Quality Requirements, respectively. He has promoted and facilitated as well a technical support from the Organization for the Prohibition of Chemical Weapons to Aqaba Special Economic Zone Authority (ASEZA) to help in making world using chemistry in peace and having the world free of chemical weapons. He participated in many non-formal education and youth activities across the Mediterranean region bridging the gap between cultures and enhancing mutual understanding. Late 2013, Dr. Ala' Al-Subeihi, one of his colleagues at ASEZA, introduced him to the project of Botanicals Risk Assessment (BRAMA), under the umbrella of the European Neighborhood and Partnership Instrument Cross Border Cooperation in the Mediterranean (ENPI-CBCMED). The BRAMA project promotes for a PhD for a group of researchers regarding risk assessment of botanicals, and guide them to official recognition through the highest European standards (registration as European toxicologists). During his PhD,

he followed a number of postgraduate courses in toxicology required for registration as a toxicologist with the Netherlands Society of Toxicology and attended conference of 3R's Denmark Symposium.



List of publications

- Al-Malahmeh, A. J., A. M. Alajlouni, J. Ning, S. Wesseling, J. Vervoort and I. M. C. M. Ivonne (2017c). "Determination and risk assessment of naturally occurring genotoxic and carcinogenic alkenylbenzenes in nutmeg-based plant food supplements." *J. Appl. Toxicol.*
- Al-Malahmeh, A., A. M. Al-ajlouni, S. Wesseling, J. Vervoort and I. M. C. M. Rietjens (2017b). "Determination and risk assessment of naturally occurring genotoxic and carcinogenic alkenylbenzenes in basil-containing sauce of pesto." *Toxicology Reports* 4: 1-8.
- Al-Malahmeh, A. J., A. M. Alajlouni, S. Wesseling, A. E. Soffers, A. Al-Subeihi, R. Kiwamoto, J. Vervoort and I. M. C. M. Rietjens (2017a). "Physiologically based kinetic modeling of the bioactivation of myristicin." *Archives of toxicology* 91(2): 713-734. from <http://www.ncbi.nlm.nih.gov/pubmed/27334372>.
- Alajlouni, A. M., A. J. Al-Malahmeh, M. Kallib, S. Wesseling, J. Vervoort and R. I. M.C.M. (2017). "Risk assessment of combined exposure to alkenylbenzenes through consumption of plant food supplements containing parsley and dill." *Journal Food Additives & Contaminants: Part A*.
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Overview of completed training activities

Discipline specific activities:

- International symposium (2015)
- Molecular toxicology (2014)
- Cell toxicology (2014)
- Organ toxicology (2015)
- Epidemiology (2014)
- Ecotoxicology (2014)
- Laboratory animal science (2014)
- Pathobiology (2014)
- Risk assessment (2014)
- Immunotoxicology (2014)
- Medical and Forensic (2014)

General Courses:

- VLAG PhD week (2014)
- PhD competence assessment (2014)
- PhD symposium (2015)
- Analytical chemistry skills (2014)
- Measurement science and statistics (2014)

Optional:

- Preparation of research proposal
- General toxicology
- Attending scientific presentation at toxicology
- PBK modelling

Approved by the graduate school VLAG

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