Physiologically based biokinetic (PBBK) modeling and validation of dose-, species-, interindividual- and matrix dependent effects on the bioactivation and detoxification of safrole

Erryana Martati 2013

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

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Erryana Martati

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M. J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 30 October 2013
at 1:30 p.m. in the Aula.

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PhD thesis, Wageningen University, Wageningen, NL (2013)
With references, with summaries in Dutch and English

ISBN 978-94-6173-745-8

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CHAPTER 1

General introduction

Short introduction and aim of the thesis

Safrole, 4-allyl-1,2-methylenedioxybenzene, is a constituent of spices like nutmeg, mace and star anise and of some food products to which safrole containing spices are added such as pesto sauce, cola beverages, Bologna sausages and Vienna sausages (Siano *et al.*, 2003). In addition, safrole containing spices are used as source of natural flavouring and as traditional medicine for illnesses related to the nervous and digestive system (Leela, 2008). Current European Council Directive (EC) on food flavouring regulation No 1334/2008 has prohibited the use of safrole as such in human foods and restrictions have been set on the concentration of safrole in foods that are prepared with safrole containing flavourings or food ingredients with flavouring properties (European Commission, 2008). In the U.S. already in 1960 the U.S. Food and Drug Administration (FDA) prohibited the use of pure safrole and sassafras oil (content of safrole >80%) in food (FDA Ban 21 CFR 189, 180; revised April 1 2008) due to the potential carcinogenicity of safrole as demonstrated in rodent studies (Borchert *et al.*, 1973; Miller *et al.*, 1983; Wislocki *et al.*, 1977).

Several risk assessments have been performed to evaluate the safety of human exposure to safrole. In 1997, the Committee of Experts on Flavouring Substances (CEFS) of the Council of Europe evaluated safrole and concluded that safrole is a weak hepatocarcinogen in experimental animal studies but also a genotoxic and a transplacental carcinogen, and that efforts should be made to reduce its consumption through foods and beverages as much as possible (Council of Europe, 1997). The Scientific Committee on Food (SCF) of the European Union (EU) concluded in their evaluation that safrole is genotoxic and carcinogenic and that reductions in the exposure and restriction in the use levels are indicated (SCF, 2002). These opinions are all based on carcinogenicity data from rodent studies because adequate human data were not available. At present, discussions are ongoing on how to translate the results from animal bioassays at high dose levels of the pure compound to the risk for the human population exposed to safrole at relatively low levels via dietary intake. This translation of cancer risk from the animal data to humans at relevant dietary intake levels needs a better understanding of species dependent, dose dependent, and interindividual differences in bioactivation and detoxification of safrole and the possible influence of the food matrix on these processes.

The aim of this thesis was to obtain insight into the effects of dose-, species-, interindividual- and matrix dependent differences on the bioactivation and detoxification of safrole using physiologically based biokinetic (PBBK) modeling.

Exposure to safrole

Although use of safrole has been banned in the EU and U.S., intake of safrole at very low levels occurs in daily life via the diet from consumption of spices containing safrole such as nutmeg, mace, star anise, cinnamon and black pepper and from food products to which these spices are added such as cola beverages, Bologna sausages and Vienna sausages (Siano *et al.*, 2003). The content of safrole in essential oils of nutmeg, mace, star anise and black pepper is 2.33, 1.05, 0.14, 0.14 and 0.1% (JECFA, 2009). The content of safrole in Bologna and Vienna sausages was determined to amount to 2 and 0.2 mg/kg of product, respectively which can most likely be ascribed to nutmeg addition (Siano *et al.*, 2003). The intake of safrole may also result from consumption of soft drinks which may contain safrole up to 3-5 times the use limit of 1 µg/mL (Choong and Lin, 2001). Furthermore, exposure to safrole occurs in some countries in Southeast Asia as a result of the popular habit of chewing a betel quid containing *Piper betle* (containing 15 mg/g fresh weight of safrole), a habit that was linked to the incidence of oral squamosa cell carcinoma (Chen *et al.*, 1999; Hwang *et al.*, 1992; Ko *et al.*, 1995).

The average daily intake of safrole from consumption of foods that contain safrole was estimated to be 0.3 mg/day corresponding to 5 µg/kg body weight (bw)/day for a 60 kg person (SCF, 2002). This value was based on a selection of 28 food categories to which safrole could still be added at the time of the evaluation. Given current regulations that restrict safrole use in food, this average daily intake may be lower at present, although recent estimates from other assessment bodies report comparable intakes. The Joint FAO/WHO Expert Committee on Food Additives (JECFA), at the sixty-ninth meeting, estimated the intake of safrole from food consumption to amount to 569 µg/person/day (equivalent to 9.5 µg/ kg bw/day for a 60 kg person) in the U.S. based on the highest reported levels of volatile oil in spices and the highest reported safrole concentration in the oil (JECFA, 2008). From EU import data of nutmeg, mace and its essential oils, the

maximum daily intake of safrole was estimated to be 879 µg/person (equivalent to 14.7 µg/ kg bw/day for a 60 kg person) (JECFA, 2008). The FDA estimated the intake of safrole from only essential oil of nutmeg and mace to be 4 µg/kg bw/day (SCOGS, 1973).

Safrole metabolism

Upon oral intake, safrole is rapidly absorbed from the gastrointestinal tract by passive diffusion (Fritzh, 1975). Biotransformation of safrole occurs mainly in the liver (Benedetti *et al.*, 1977; Klungsøyr and Scheline, 1983). The metabolism of safrole including its bioactivation and detoxification pathways is presented in Figure 1.1. The metabolic pathways of safrole include oxidative dealkylation of the methylenedioxy group resulting in 1,2-dihydroxy-4-allylbenzene (DHAB) which is the major metabolite of safrole in rat, guinea pig and man exposed to safrole (Benedetti *et al.*, 1977; Chang *et al*., 2002; Klungsøyr and Scheline, 1982; Stillwell *et al.*, 1974). This metabolite may be further oxidized to an *o*-quinone metabolite, 4-allyl-*o*-quinone, which isomerizes non-enzymatically to a more electrophilic *p*-quinone methide (Bolton *et al.*, 1994). DHAB can be excreted following conjugation at its hydroxyl moieties through sulfation and/or glucuronidation prior to oxidation to the quinone/quinone methide or upon GSH conjugation of its quinone/quinone methide which has been observed to occur in rat hepatocytes (Nakagawa *et al.*, 2009). However, GSH conjugation was a minor pathway compared with the sulfation and/or glucuronidation of DHAB (Nakagawa *et al.*, 2009). A small amount of 3′-hydroxysafrole as conjugated form has also been detected in the urine of rats exposed to safrole, but not in the urine of humans exposed to safrole (Benedetti *et al.*, 1977). This metabolite is formed upon the direct hydroxylation of the alkyl side chain at the 3′ position and is subsequently conjugated with glycine to give 3,4-methylenedioxybenzoyl glycine and 3,4 methylenedioxy-cinnamoyl glycine which are excreted in the urine (Boberg *et al.*, 1986). Epoxidation of the double bond of the allyl side chain results in 2′,3′-safrole oxide which is rapidly hydrolysed by microsomal epoxide hydrolase forming 2′,3′-dihydroxysafrole (Ioannides *et al.*, 1981; Klungsøyr and Scheline, 1983). The major bioactivation pathway of safrole is initiated by formation of the proximate carcinogenic metabolite 1′-hydroxysafrole in a reaction catalysed by cytochromes P450, with P450 2A6,

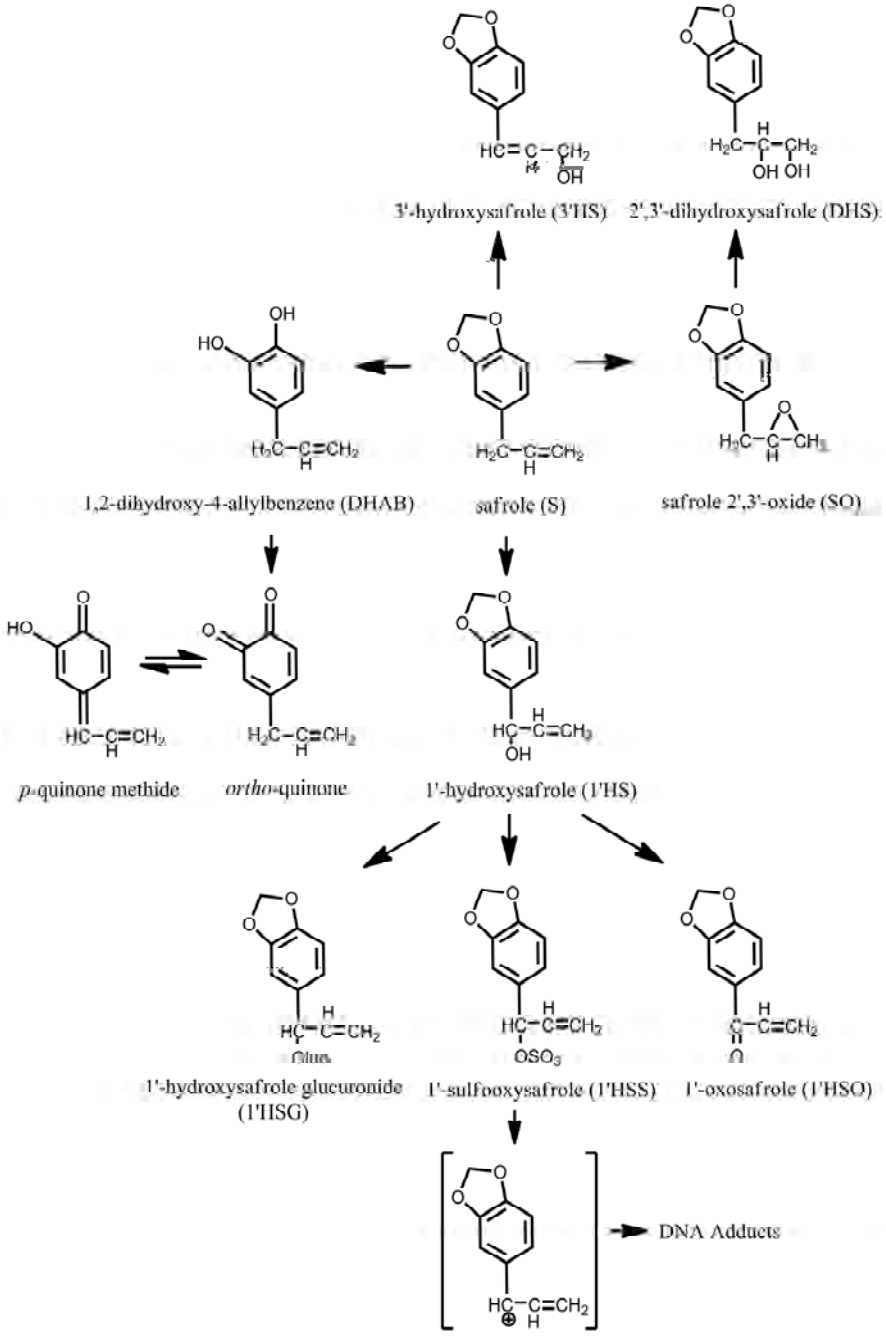

Figure 1.1. Biotransformation pathways of safrole.

P450 D6.1, P450 2C9, P450 2C19 and P450 2E1 being the main P450 enzymes involved in human (Jeurissen *et al.*, 2004; Liu *et al.*, 2004; Ueng *et al.*, 2004). 1′-Hydroxysafrole subsequently undergoes several further reactions. One of these reactions is further bioactivation to the ultimate carcinogen 1′-sulfooxysafrole catalyzed by sulfotransferases (SULTs). Detoxification of 1′-hydroxysafrole may result from the conjugation reaction catalyzed by UDP-glucuronosyltransferases (UGTs) resulting in 1′-hydroxysafrole glucuronide which is excreted in the urine in rodents and humans. Another reaction of 1′-hydroxysafrole is its oxidation forming 1′-oxosafrole which was detected in small amounts as conjugated form in the bile of rats accounting for 6.5% of the dose of 1′-hydroxysafrole injected intraperitoneal (i.p) to rats (Fennell *et al.*, 1984). 1′-Oxosafrole is subsequently conjugated with glutathione forming 3′-(glutathion-*S*-yl)-1′-oxo-2′,3′-dihydrosafrole which can be converted to the corresponding mercapturic acid 3′-(*N*-acetylcystein -*S*-yl)-1′-oxo-2′,3′-dihydrosafrole (Fennell *et al.*, 1984).

Carcinogenicity of safrole

Long term carcinogenicity studies of safrole in rodents have been performed. Table 1.1 presents an overview of the available carcinogenicity data from long term studies with safrole in mice and rats. Wislocki *et al*. (1977) employed oral administration of safrole via the diet to rats for 51 weeks. In this study, the incidence of hepatomas was increased (17%) at a dose of 200 mg safrole/kg bw/day. Rats exposed to 68 mg safrole/kg bw/day and controls showed no hepatomas. Three independent studies using mice and rats were reported in the publication by Borchert *et al.* (1973). In study 1, administration of safrole via the diet to male CD-1 mice at dose levels of 390 and 488 mg safrole/kg bw/day for 56 weeks resulted in a significant increase in the incidence of hepatocellular carcinomas, by 44 and 53%, respectively, compared to controls (0%). In study 2, rats were administered safrole via the diet at dose levels of 82 and 138 mg/kg bw/day for 47 weeks and this resulted in the formation of hepatocellular carcinomas but not at levels that were significantly different compared to the levels in controls. In study 3, increasing levels of hepatocellular carcinomas were observed in male CD-1 mice dosed by subcutaneous (s.c) injection with 9.45 µmol of safrole (given in four injections on days 1, 7, 14, and 21 of age to preweanling mice) but no hepatocellular carcinomas formed in female CD-1 mice

(Borchert *et al.*, 1973). When safrole was administered to post weaning mice, a significant increase in hepatocellular tumors was observed in females (48%) but not in males (8%). Male offspring (34%) nursed by mothers treated treated with safrole during lactation had hepatocelluler tumors but not female offspring (Vesselinovitch *et al.*, 1979). The results suggested that safrole or its metabolites may cross the placenta to the fetuses and be transferred from lactating mothers exposed to safrole to the offspring through the milk (Vesselinovitch *et al.*, 1979).

Table 1.1. Overview of carcinogenicity data from rodent studies with safrole reported in literature

Species	Sex	Route of administration	Duration of exposure	Sacrifice	Dose	# of animals with liver tumor or hepatocellular carcinoma/#animals	Incidence (%)	References
Mice B6C3F1	M	Transplan-cental	18 days (12,14,16,18 days of gestation)	92 wk	0	3/100	3	Vesselinovitch *et al.* (1979)
					Total dose 500 mg/kg bw	2/63	3.2	
	F				0	0/98	0	
					Total dose 500 mg/kg bw	0/71	0	
	M	gastric intubation	90 wk (twice weekly)		0	3/100	3	
					120 mg/kg bw/day	4/60	8	
	F				0	0/98	0	
					120 mg/kg bw/day	22/60	48	
	M	via milk	3 wk (2 days interval)		0	3/100	3	
					Total dose 1500 mg/kg bw	28/85	34	
	F				0	0/98	0	
					Total dose 1500 mg/kg bw	2/80	2.5	
Rat CD1	M	diet	74 wk	96wk	0	0/18	0	Wislocki *et al.* (1977)
					0.22% (68 mg/kg bw/day)	0/18	0	
			96 wk		0	0/15	0	
					0.5% (200 mg/kg bw/day)	3/18	17	
Mice CD1	F	diet	52 wk	78wk	0	0/30	0	Miller *et al.* (1983)
					0.5% (176 mg/kg bw/day)	21/30	70	
	F			86 wk	0	0/50	0	
					0.25% (80 mg/kg bw/day)	34/47	72	
					0.5% (160 mg/kg bw/day)	39/49	80	
Mice CD1	M	diet	56 wk	70 wk	0	4/50	8	Borchert *et al.* (1973)
					0.4% (390 mg/kg bw/day)	11/25	44	
					0.5% (488 mg/kg bw/day)	8/15	53	
	M	s.c injection	3 wk (at day 1,7,14,21)	56 wk	0	4/36	11	
					Total 9.45 µmol	20/35	57	
	F				0	0/31	0	
					Total 9.45 µmol	0/27	0	
Rat (SD)	M	diet	47 wk	52 wk	0	0/18	0	
					0.3% (82 mg/kg bw/day)	0/18	0	
					0.5% (138 mg/kg bw/day)	1/18	6	

Note : M: male; F: female; i.p: intraperitoneal; s.c: subcutaneous; s.t: stomach tube; wk: week

In addition, also several carcinogenicity studies which administered the proximate carcinogenic metabolite of safrole, 1′-hydroxysafrole, were performed in both mice and rats through various routes of administration. Table 1.2 presents the data of these carcinogenicity studies with 1′-hydroxysafrole. In general, the results show that the 1′-hydroxy metabolite of safrole also induces hepatocellular tumors in rodent studies. Male rats dosed with 1′-hydroxysafrole at 77 mg/kg bw/day for 43 weeks and 100 mg/kg bw/day for 73 weeks showed incidences of hepatocellular carcinoma of 40 and 90%, respectively (Wislocki *et al.*, 1977). Boberg *et al*. (1983) showed that oral dose levels of 125 mg/kg

Table 1.2. Overview of carcinogenicity data from rodent studies with 1′-hydroxysafrole reported in literature

Species	Sex	Route of administration	Duration of exposure	Sacrifice	Dose	# of animals with liver tumor or hepatocellular carcinoma/#animals	Incidence (%)	References
Mice CD-1	F	diet	52 wk	70 wk	0	0/32	0	Boberg *et al.* (1983)
					125 mg/kg bw/day	17/29	60	
					260 mg/kg bw/day	25/32	78	
Rat CD	M	diet	73 wk	96 wk	0	0/18	0	Wislocki *et al.* (1977)
			43 wk		0.25% (77 mg/kg bw/day)	7/18	40	
			73wk		0.55% (100 mg/kg bw/day)	16/18	90	
Mice B6C3F	M	i.p	Injection on day 8, 12	44 wk	0	4/33	12	Boberg *et al.* (1983)
					0.05 µmol/g bw/day	12/34	36	
					0.1 µmol/g bw/day	31/36	86	
					0.2 µmol/g bw/day	32/33	97	
Mice CD-1	M	i.p	Injection on day 1,8,15,22	52 wk	0	7/46	15	Miller *et al.* (1983)
					4.72 µmol /g bw/day	30/46	65	
Mice B6C3F1	M	s.c	Injection on day1,8,15 and 22)	52 wk	0	5/35	14	
					3.75 µmol/ g bw/day	24/26	92	
Rat Fischer	M	s.c	Injection twice weekly (10 wk)	104 wk	0	0/20	0	
					Total dose 2 mmol/rat	11/20	55	

Note : M: male; F: female; i.p: intraperitoneal; s.c: subcutaneous; s.t: stomach tube; wk: week

bw/day and 260 mg/kg bw/day of 1′-hydroxysafrole given to female mice caused 60 and 78% increase in the hepatoma incidence as compared to control, respectively (Boberg *et al.*, 1983).

DNA adduct formation and genotoxicity of safrole

Safrole was not mutagenic in the Salmonella reverse mutation assay (Ames test) (Swanson *et al.*, 1979). 1′-Hydroxysafrole was mutagenic for *S. Typhimurium* strain TA 100 (Swanson *et al.*, 1979). Bioactivation of safrole by sequential 1′-hydroxylation and sulfation to 1′-sulfooxysafrole results in a reactive intermediate capable of forming protein, RNA and DNA adducts, the latter of which may lead to carcinogenesis (Randerath *et al.*, 1984; Boberg *et al.*, 1983; Miller *et al.*, 1983). DNA adducts induced by safrole and its reactive metabolites 1′-hydroxysafrole and 1′-sulfooxysafrole have been characterized. Phillip *et al*. (1981) have identified DNA adducts formed in the liver of adult female CD-1 mice after administration of ^{3}H-labelled 1′-hydroxysafrole. The major adduct was characterized as N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine (S-3′-N^2-dGuo). Minor DNA adducts detected were N^2-(safrol-1′-yl)-2′-deoxyguanosine (S-1′-N^2-dGuo) and N^6-(*trans*-isosafrol-3′-yl)-2′-deoxyadenosine (S-3′-N^6-dAdo) (Phillips *et al.*, 1981). Daimon *et al.* (1998) isolated four DNA adducts from the liver of rats exposed to safrole. Two DNA adducts were identified as S-3′-N^2-dGuo and S-1′-N^2-dGuo which are similar to the ones reported by Phillips *et al*. (1981) and Randerath *et al*. (1984), while two other safrole-DNA adducts remained unidentified (Daimon *et al.*, 1998). Figure 1.2 presents the schematic pathway for bioactivation of safrole and the nature of the DNA adducts formed. The safrole metabolite 2′,3′-safrole oxide is also an electrophilic metabolite that was shown to react with DNA bases to form DNA adducts in vitro but these adducts were not detected in mice exposed to safrole (Qato and Guenthner, 1995). This absence of DNA adduct formation by 2′,3′-safrole oxide in vivo may be due to rapid detoxification of 2′,3′-safrole oxide by glutathione S-transferase and epoxide hydrolases (Ioannides *et al.*, 1981; Klungsøyr and Scheline, 1983; Guenthner and Luo, 2001).

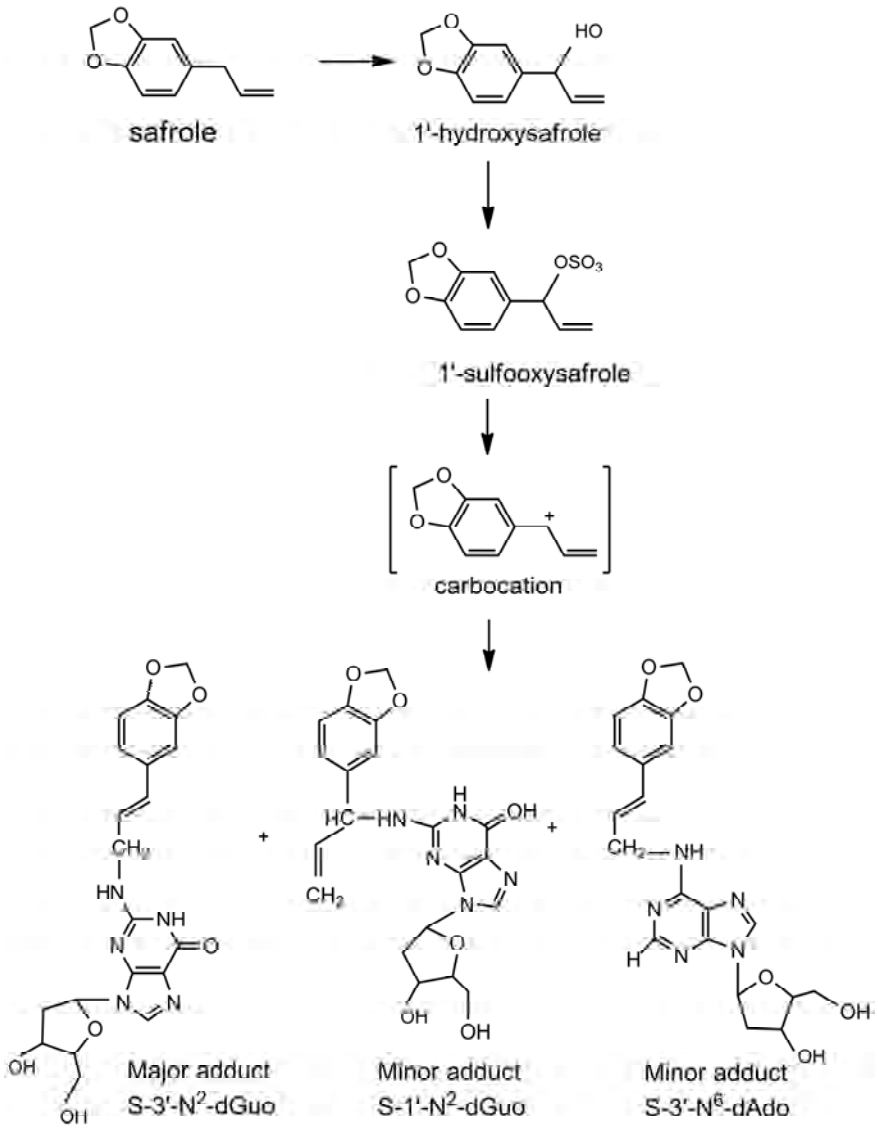

Figure 1.2. Bioactivation pathways of safrole and the structure of DNA adducts as identified in literature (Chung *et al.*, 2008; Daimon *et al*., 1998; Randerath *et al*., 1984; Phillips *et al*., 1981). S-3′-N^2-dGuo= N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine, S-1′-N^2-dGuo= N^2-(safrol-1′-yl)-2′-deoxyguanosine and S-3′-N^6-dAdo= N^6-(*trans*-isosafrol-3-yl)-2′-deoxyadenosine.

Studies with hepatocytes isolated from F344 rats exposed to safrole by gastric intubation showed that safrole was able to induce chromosome aberrations, sister chromatid exchanges (SCEs) and DNA adducts (Daimon *et al.*, 1998). Five repeated administrations of 125 and 250 mg/kg bw of safrole increased the aberrant cells in the liver dose dependently. Both administration of a single dose (100 - 500 mg/kg bw safrole) and five repeated doses (62.5 and 125 mg/kg bw safrole) induced SCEs in the liver (Daimon *et al*., 1998). In another in vivo study, it was reported that adduct formation in the female mice liver was approximately linear with the dose of safrole down to the lowest dose tested in a range from 1-10 mg safrole/mouse with no indication of a threshold. Only at the highest concentration of safrole tested (10 mg/mouse), a slight deviation from linearity was observed (Gupta *et al.*, 1993).

Formation of DNA adducts upon exposure to safrole was also observed in humans; in a sample of oral tissue obtained from an oral cancer patient having a habit of betel quid chewing the presence of DNA adducts identified as S-3′-N^2-dGuo was reported (Chung *et al.*, 2008).

Cancer risk assessment

The presence of safrole in herbs, spices or processed foods cannot be avoided resulting in a certain level of daily exposure. Because safrole is both genotoxic and carcinogenic, the prediction of the resulting cancer risk at low dose levels representing realistic human dietary intake is needed. At present there are different approaches that are available for assessing the risk of substances that are both genotoxic and carcinogenic (EFSA, 2005). There is no international harmonised approach for risk assessment of compounds that are both genotoxic and carcinogenic. The difficulties in risk assessment of compounds that are both genotoxic and carcinogenic originate from the fact that the carcinogenic process induced by genotoxicity is normally regarded to have no threshold, thereby, eliminating the possibility for definition of a safe level of exposure (EFSA, 2005). The mutagenic carcinogenic process may be initiated already upon exposure at very low levels and therefore a NOAEL cannot be identified. A principle of ALARA (as low as reasonably achievable) was used in risk management of compounds that are both genotoxic and

carcinogenic but this approach does not enable a risk manager to assess the urgency and the extent of the risk reduction measures needed (EFSA, 2005). The extrapolation of tumor data obtained at high dose levels in rodent studies to the risk for humans at realistic low dietary exposure levels is particularly important. This implies that animal data from bioassays using high dose levels have to be translated to the human situation with very low levels of intake. For the extrapolation from high doses in animal studies to lower exposure in human, some mathematical models are available. A Virtual Safe Dose (VSD), a dose which provides a response equivalent to 1 extra tumor case in 1.000.000 upon lifetime exposure, can be estimated by extrapolation from the dose causing a certain cancer incidence to the lower dose range estimating the dose that would give this incidence of 1 in a million upon life time exposure. This implies extrapolation far outside the experimental dose and tumor incidence range and an outcome for the VSD that depends very much on the extrapolation model applied (EFSA, 2005). Linear extrapolation for assessing the risk of chemicals that are genotoxic and carcinogenic and calculating the VSD is criticized because it is unknown whether linear extrapolation actually reflects the underlying biological processes and because it does not take species differences into account (EFSA, 2005). The Margin of Exposure (MOE) approach was recently proposed by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA) as an alternative approach for the risk assessment of compounds that are both genotoxic and carcinogenic (EFSA, 2005). The MOE is based on the available animal dose-response data, without extrapolation outside the experimental data range, and on human intake data. The MOE approach needs a reference point which can be taken from the dose response data of the animal experiments and should represent a dose causing a low but measurable cancer response. The MOE can be calculated as the ratio between the reference point (e.g. $BMDL_{10}$) (the lower confidence limit of the benchmark dose causing 10% extra tumor incidence above background levels (BMD_{10})) and the estimated daily dietary human intake (EDI). An MOE of 10,000 or higher is considered as a low priority for risk management (EFSA, 2005). This MOE of 10,000 is applied to adequately allocate various uncertainties; taking into account a factor 100 for species differences and human variability in the basic processes of toxico-kinetics and toxico-dynamics, a factor 10 for

interindividual human variability in cell cycle control and DNA repair, and a factor 10 for uncertainties in the shape of the dose-response curves outside the observed dose range (EFSA, 2005).

Overall, it is important to note that the MOE does not provide a quantitative risk assessment, but especially provides a tool to set priorities in risk management. This implies that new approaches for risk assessment of compounds that are both genotoxic and carcinogenic are required. Quantitative risk assessment of a compound that is genotoxic and carcinogenic requires extrapolation from high to low dose, from one exposure route to another and from one exposure duration to another which can all be performed using a physiologically based biokinetic (PBBK) approach. In addition, extrapolation from animal toxicity data cannot assure human safety without understanding of the species dependent differences in kinetics of absorption, distribution, metabolism and excretion (ADME) of the compound of interest. PBBK modeling provides a powerful method for increasing the accuracy of the extrapolation because it facilitates extrapolation from animal species to human, from high doses to low doses and from one exposure route or time frame to another (Clewell, 1995). An accurate assessment of risk associated with exposure to a hazardous chemical should be based on all the biologically relevant mechanistic data (Andersen and Krishnan, 1994). Biologically based PBBK models provide a method for increasing the accuracy of these extrapolations (Clewell, 2010).

Physiologically based biokinetic (PBBK) modeling

Physiologically based biokinetic (PBBK) models are mathematical descriptions of the ADME characteristics of a chemical and its metabolites in the species and organs of interest. The mathematical descriptions include physiological (e.g. blood flow), physicochemical (e.g. partition coefficients) and biochemical (e.g. metabolic rates) parameters. PBBK models consist of many compartments representing individual target tissues (e.g. liver, kidney, lung) or non-target tissues lumped together in one compartment (e.g. slowly perfused tissues such as muscle and skin or richly perfused tissues such as brain and spleen) (Punt *et al.*, 2007).

Exposure to carcinogenic compounds leads to absorption and distribution of the compounds or their reactive metabolites to the target tissues. These reactive metabolites may alter the chemical structure of the DNA of the target tissues which may initiate tumor formation. Using the PBBK models, the dose of the reactive metabolites reaching the target tissues can be predicted. Quantitative information on the concentration of the reactive form of a compound in the target tissue of interest in different species provides a better basis for species dependent extrapolation in risk assessment (Andersen and Krishnan, 1994). PBBK models have been used in risk assessment of various carcinogenic compounds. One example of the application of PBBK models in risk assessment is the development of a PBBK model for inhaled styrene by Ramsey and Andersen (Ramsey and Andersen, 1984). The model was evaluated with a set of data of rats and human and used for dose route, dose level and interspecies extrapolation for time dependent changes in tissue and blood levels of styrene. The obtained PBBK models adequately described the blood and organ concentrations of styrene for intravenous (iv), inhalation and intragastric doses of styrene and this information was subsequently used for route to route extrapolations in the risk assessment. Another example of the application of a PBBK model in risk assessment is the PBBK model for vinyl chloride (Clewell *et al*., 1995). The PBBK models developed described the uptake, distribution and metabolism of vinyl chloride in mouse, rat, hamster and human following inhalation or oral exposure. The mode of action for carcinogenicity of vinyl chloride appears to be DNA adduct formation by its reactive metabolite chloroethylene epoxide. The results showed that the PBBK model based predictions for vinyl chloride were in good agreement with risk estimates based on inhalation studies with mice and rats facilitating dose-response extrapolation across species. Furthermore, the risks estimated from dietary administration were in accordance with those derived from the inhalation route (Clewell *et al.*, 1995).

Food matrix effects

Another factor that should be take into account in risk assessment for food-born compounds that are genotoxic and carcinogenic is the effect of the food matrix. Safrole has been demonstrated to be carcinogenic in *in vivo* studies. However, all of these studies have been done using high doses of the pure compound without taking matrix effects into

considerations. In a realistic human situation, exposure to safrole occurs mainly via consumption of nutmeg, mace and their essential oils and/or food products containing these spices or essential oils (SCF, 2002). Possible modulating effects of other compounds present in the herbs or spices or in other parts of the diet on the bioavailability and/or on the cytochrome P450 and or SULT-catalyzed bioactivation of safrole may occur and may have to be taken into account in the risk assessment.

Some spices were found to inhibit cytochrome P450 2C9, one of the cytochrome isoforms found to be involved in the metabolism of safrole to 1′-hydroxysafrole (Jeurissen *et al.*, 2004; Kimura *et al.*, 2010; Ueng *et al.*, 2004). Furthermore for the related alkenylbenzene estragole present in basil it was recently demonstrated that a methanolic basil extract and its isolated constituent nevadensin inhibited the sulfation and resulting DNA adduct formation of the proximate carcinogenic 1′-hydroxyestragole metabolite in studies using rat and human S9 protein, the hepatoma cell line HepG2 and/or rat hepatocytes (Alhusainy *et al.*, 2010; Jeurissen *et al.*, 2008). Moreover, an in vivo study with Sprague-Dawley rats showed that co-administration of estragole with nevadensin, at dose levels with a ratio reflecting their actual presence in basil, resulted in a significant 36% reduction in the levels of N^2-(*trans*-isoestragol-3′-y1)-2′-deoxyguanosine (E-3′-N^2-dGuo) DNA adducts formed in the liver (Alhusainy *et al.*, 2013). Thus matrix effects should preferably be taken into account in the risk assessment and studies on bioactivation and detoxification of safrole.

Objective and outline of this thesis

The aim of this thesis was to obtain insight into the effects of dose-, species-, interindividual- and matrix dependent differences on the bioactivation and detoxification of safrole using PBBK modeling.

This chapter, chapter 1, presents an introduction to the bioactivation and detoxification of safrole, safrole DNA adduct formation and the resulting tumor incidence, PBBK modeling, the importance of the matrix effects in the risk assessment of food-borne chemicals and the aim and the content of this thesis. In chapter 2, a PBBK model for safrole bioactivation and detoxification in rats is defined. Chapter 3 defines the PBBK model for safrole bioactivation and detoxification in human with a Monte Carlo simulation to predict

interindividual variation in the population as a whole. To investigate whether the food matrix modulates sulfotransferase (SULT)-catalyzed bioactivation of safrole, in chapter 4 the safrole containing spice mace is analysed for possible ingredients that may modulate safrole bioactivation. The kinetic data for interference with safrole bioactivation obtained for the thus identified mace ingredient are incorporated into the PBBK model to predict the effect of this matrix ingredient on formation of 1′-sulfooxysafrole in rat and human. An in vivo validation study in rats to evaluate the PBBK model based predictions for the effect of the mace ingredient on safrole bioactivation and DNA adduct formation in vivo is presented in chapter 5. Finally, chapter 6 summarizes the results obtained, and presents an overall discussion including future perspectives to be addressed.

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CHAPTER 2

Physiologically based biokinetic (PBBK) model for safrole bioactivation and detoxification in rats

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Based on: Chemical Research in Toxicology (2011) 24, 818-834

Abstract

A physiologically based biokinetic (PBBK) model for alkenylbenzene safrole in rats was developed using in vitro metabolic parameters determined using relevant tissue fractions. The performance of the model was evaluated by comparison of the predicted levels of 1,2-dihydroxy-4-allylbenzene and 1′-hydroxysafrole glucuronide to levels of these metabolites reported in the literature to be excreted in the urine of rats exposed to safrole, and by comparison of the predicted amount of total urinary safrole metabolites to the reported levels of safrole metabolites in the urine of safrole exposed rats. These comparisons revealed that the predictions adequately match observed experimental values. Next, the model was used to predict the relative extent of bioactivation and detoxification of safrole at different oral doses. At low as well as high doses, P450 mediated oxidation of safrole mainly occurs in the liver in which 1,2-dihydroxy-4-allylbenzene was predicted to be the major P450 metabolite of safrole. A dose dependent shift in P450 mediated oxidation leading to a relative increase in bioactivation at high doses was not observed. Comparison of the results obtained for safrole with the results previously obtained with PBBK models for the related alkenylbenzenes estragole and methyleugenol revealed that the overall differences in bioactivation of the three alkenylbenzenes to their ultimate carcinogenic 1′-sulfooxy metabolites are limited. This is in line with the generally less than 4-fold difference in their level of DNA binding in in vitro and in vivo studies and their almost similar $BMDL_{10}$ values (lower confidence limit of the benchmark dose that gives 10% increase in tumor incidence over background level) obtained in in vivo carcinogenicity studies. It is concluded that in spite of differences in the rates of specific metabolic conversions, overall the levels of bioactivation of the three alkenylbenzenes are comparable which is in line with their comparable carcinogenic potential.

Introduction

Safrole (1-allyl-3,4-methylenedioxybenzene) is an alkenylbenzene that is recognized to be a genotoxic carcinogenic agent in rodents (Borchert *et al.*, 1973; Miller and Miller, 1983; Phillips *et al.*, 1981). In rats, its hepatocarcinogenicity was reported to correlate closely with its DNA adduct formation in the liver (Miller and Miller, 1983; Phillips *et al.*, 1981). Safrole is the main component of sassafras oil, and it occurs as a trace constituent in star anise, nutmeg and black pepper and can be found in food containing these herbs such as pesto sauce, cola beverages, Bologna sausages and Vienna sausages (Siano *et al.*, 2003). The habit of chewing a betel quid which contains leaves of *Piper betle* (15 mg/g fresh weight of safrole) have been reported to result in safrole concentrations of up to 420 µM in saliva during chewing (Hwang *et al.*, 1992).

Safrole and sassafras oil were banned as food additives and flavoring agents by the FDA in 1960 because of their carcinogenic potential. The European Council Directive on food flavoring 88/388/EEC, amended by Commission Directive 91/71/EEC, limited safrole in foodstuff and beverages to 1 ppm. In 1997, the Committee of Experts on Flavoring Substances (CEFS) of the Council of Europe evaluated safrole and concluded that safrole is a weak hepatocarcinogen in experimental animal studies but also a genotoxic and a transplacental carcinogen, and that efforts should be made to reduce its consumption through foods and beverages as far as possible.

The Scientific Committee for Food of the European Union (SCF, 2002) estimated the average daily intake of safrole to be 0.3 mg/day equivalent to 5 µg/kg bw/day for a 60 kg person while the Joint FAO/WHO Expert Committee on Food Additives (JECFA) estimated the maximum dietary exposure to safrole to amount to 879 µg/person/day equivalent to 14.6 µg /kg bw/day for a 60 kg person.

The genotoxic and hepatocarcinogenic action of safrole has been correlated to the formation of its proximate carcinogenic metabolite 1′-hydroxysafrole followed by sulfation to the ultimate carcinogenic metabolite 1′-sulfooxysafrole (Borchert *et al.*, 1973; Wislocki *et al.*, 1976; Wislocki *et al.*, 1977). A second pathway for bioactivation to an electrophilic metabolite consists of the formation of 1,2- dihydroxy-4-allylbenzene, which can

subsequently be oxidized to an *o*-quinone leading to an electrophilic *p*-quinone methide intermediate (Bolton *et al.*, 1994). Figure 2.1 gives a schematic overview of the metabolism of safrole presenting these pathways for bioactivation as well as the relevant detoxification pathways. Bioactivation of safrole to 1′-hydroxysafrole is catalyzed by cytochromes P450, with P450 2A6, P450 D6.1, P450 2C9, P450 2C19 and P450 2E1 being the main P450 enzymes involved in humans (Jeurissen *et al.*, 2004; Liu *et al.*, 2004; Ueng *et al.*, 2004). Sulfation by sulfotransferases (SULTs) converts 1′-hydroxysafrole to 1′-sulfooxysafrole which is unstable and spontaneously decomposes to produce an electrophilic carbonium cation which can form covalent adducts with cellular macromolecules (Borchert *et al.*, 1973; Ioannides *et al.*, 1981; Wislocki *et al.*, 1977). A major detoxification pathway of 1′-hydroxysafrole in rats is the conjugation of 1′-hydroxysafrole with glucuronic acid catalyzed by UDP-glucuronosyltransferases (UGTs) resulting in a stable metabolite which is excreted in the urine (Benedetti *et al.*, 1977; Stillwell *et al.*, 1974). In humans, however, oxidation of the 1′-hydroxy metabolite of the related alkenylbenzene estragole appeared to be the major pathway for detoxification of 1′-hydroxyestragole (Punt *et al.*, 2009) and it remains to be established whether this species difference in detoxification of the 1′-hydroxy metabolite also occurs for safrole. In rats, the oxidation of the 1′-hydroxy metabolite may be a minor pathway (Punt *et al.*, 2008).

Small amounts of 3′-hydroxysafrole as conjugated form have also been detected in the urine of rats exposed to safrole, but not in humans (Benedetti *et al.*, 1977). The occurrence of this metabolite may arise from isomerization of 1′-hydroxysafrole during glucuronidase hydrolysis (Borchert *et al.*, 1973; Stillwell *et al.*, 1974) although others have questioned this isomerization and pointed at direct 3′-hydroxylation as a minor metabolic pathway, on the basis of the detection in urine of 3,4-methylenedioxybenzoyl glycine and 3,4-methylenedioxycinnamoyl glycine which would result from oxidation of the side chain of 3′-hydroxysafrole (Boberg *et al.*, 1986). Swift detoxification of 3′-hydroxysafrole via these pathways may explain that the levels of hepatic nucleic acid and protein adducts formed in mice upon administration of 3′-hydroxysafrole were much lower than those obtained upon administration of 1′-hydroxysafrole (Boberg *et al.*, 1986).

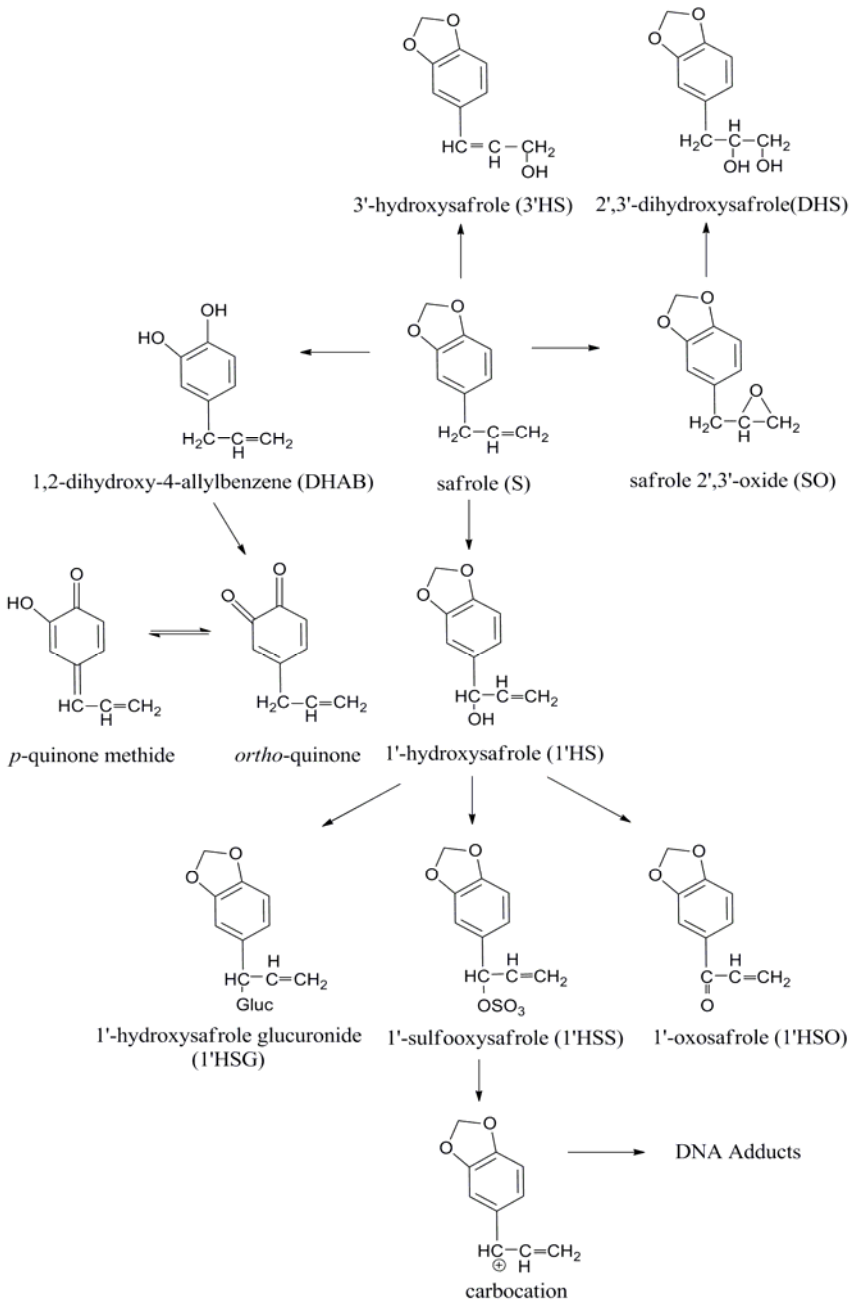

Figure 2.1. Proposed biotransformation pathways of safrole.

Oxidative dealkylation of the methylenedioxy group of safrole results in the formation of 1,2-dihydroxy-4-allylbenzene as a major metabolite in rats, guinea pigs and humans (Benedetti *et al.*, 1977; Chang *et al.*, 2002; Klungsøyr and Scheline, 1982; Stillwell *et al.*, 1974), which may be converted by oxidation to quinone metabolites such as 4-allyl-*o*-quinone which isomerizes nonenzymatically to a more electrophilic *p*-quinone methide (Bolton *et al.*, 1994). In vitro studies showed that 1,2-dihydroxy-4-allylbenzene induced chromosome aberration in Chinese hamster ovaries (CHO-K1) in a dose dependent manner (10-50 µM) and increased the frequency of micronuclei up to 3 times at concentration of 40 µM (Lee-Chen *et al.*, 1996). However, 1,2-dihydroxy-4-allylbenzene can be excreted following conjugation at its hydroxyl moieties prior to oxidation to the quinone/quinone methide or upon GSH conjugation of its quinone/quinone methide which has been observed to occur in rat hepatocytes (Nakagawa *et al.*, 2009). Thus, bioactivation of 1,2-dihydroxy-4-allylbenzene to its quinone/quinone methide metabolites may be observed especially in systems without adequate conjugation possibilities. When such conjugation reactions are adequate, such as in vivo, possible adverse effects of 1,2-dihydroxy-4-allylbenzene are likely to be limited.

Another metabolic pathway of safrole is epoxidation of the double bond in the allyl side chain yielding 2′,3′-safrole epoxide which is susceptible to hydrolysis by microsomal epoxide hydrolase forming 2′,3′-dihydroxysafrole (Ioannides *et al.*, 1981; Klungsøyr and Scheline, 1983).

To predict the extent of bioactivation and detoxification of safrole at low dose levels based on experimental data obtained at relatively higher dose levels, it is important to take nonlinear effects in toxicokinetics into account when extrapolating from high to low doses. The objective of the present study was to build a physiologically based biokinetic (PBBK) model providing insight into possible dose dependent changes in safrole metabolism with emphasis on detoxification and bioactivation pathways. Kinetics for relevant P450 mediated oxidation reactions of safrole and conjugation reactions of 1′-hydroxysafrole were determined in vitro using tissue fractions of organs that were found to be capable of metabolizing safrole or 1′-hydroxysafrole. The kinetic data obtained in vitro were used to build a PBBK model for rats which was used to estimate the time and dose dependent

formation of the proximate and ultimate carcinogenic metabolites of safrole, 1′-hydroxysafrole and 1′-sulfooxysafrole in vivo. The performance of the model was evaluated by comparison of the predicted level of metabolites formation to the reported levels of metabolites excreted in the urine of Wistar rats (Klungsøyr and Scheline, 1983) and male Sprague-Dawley rats exposed at different levels of safrole (Benedetti *et al.*, 1977). The metabolites included in this comparison were 1,2-dihydroxy-4-allylbenzene, 1′-hydroxysafrole glucuronide and the total urinary excretion of safrole metabolites.

Materials and methods

Caution : Safrole, 1′-hydroxysafrole, 1,2-dihydroxy-4-allylbenzene and 1′-oxosafrole are carcinogenic compounds which should be treated carefully.

Materials. Pooled male rat liver microsomes and S9 from Sprague-Dawley were purchased from BD Gentest (Worburn,U.S.). Pooled male rat lung, kidney and small intestinal microsomes (Sprague-Dawley) were obtained from BioPredic International (Rennes, France). Safrole (purity 97%), 2′,3′-dihydroxysafrole (purity 99%), Tris (hydroxymethyl)aminomethane, alamethicin (from *Trichoderma viride*), L-ascorbic acid, uridine 5′-diphosphoglucuronide acid (UDPGA) and 3,4-(methylenedioxy) cinnamic acid were purchased from Sigma-Aldrich (Steinheim, Germany). β-Glucuronidase and reduced (NADPH) and oxidized (NAD^+) β-nicotinamide adenin dinucleotide phosphate were obtained from Roche Diagnostics (Mannheim, Germany). Glutathione reduced (GSH) and dimethyl sulfoxide (DMSO) were purchased from Acros Organic (New Jersey, U.S.). 3′-Phosphoadenosine-5′-phosphosulphate (PAPS) was obtained from Fluka (Buchs, Switzerland). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate trihydrate, acetic acid (glacial) and magnesium chloride were purchased from VWR Merck (Darmstadt, Germany). Chromatography grade acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands). 1′-Hydroxysafrole was synthesized as described previously (Jeurissen *et al.*, 2004) and purified using a preparative thin-layer chromatography method with a mobile phase system of a mixture of petroleumbenzene and ethylacetate (50:50). The purity of 1′-hydroxysafrole was determined by HPLC to be about 99%. 3′-Hydroxysafrole was synthesized from 3,4-(methylenedioxy)cinnamic acid as

described previously (Angle *et al.*, 2008) and purified using a preparative thin-layer chromatography method with a mobile phase system of petroleumbenzene and ethylacetate (50:50). The purity of 3′-hydroxysafrole was determined by HPLC and amounted to about 97%. 1′-Oxosafrole was synthesized from 1′-hydroxysafrole on the basis of the method used for 1′-oxoestragole (Wislocki *et al.*, 1976). 1,2-Dihydroxy-4-allylbenzene was synthesized as described previously (Bolton *et al.*, 1994). The purity of 1,2-dihydroxy-4-allylbenzene was determined by HPLC to be about 95%.

P450 mediated oxidation of safrole

Metabolites formed during P450 mediated oxidation of safrole were identified and quantified upon the incubation of safrole with pooled male rat (Sprague-Dawley) liver, lung and kidney microsomes in the presence of NADPH. The incubations were performed at 37°C containing 1 mg/mL of microsomal protein, 3 mM NADPH, 1 mM ascorbic acid in 0.2 M Tris-HCl pH 7.4. After 1 min of preincubation at 37°C the reactions were started by adding safrole from a 100 times concentrated stock solutions in DMSO. To identify which organs were involved in safrole metabolism the incubations were first performed at concentration of 1000 µM. After 20 min the reactions were terminated by adding 40 µL of ice-cold acetonitrile to the total incubation volume of 160 µL. All samples were centrifuged for 5 min at 16000 g and frozen at -20°C until analysis by HPLC. For determination of the kinetic constants for formation of the various P450 mediated oxidation products of safrole the same conditions were used, with the final concentration of safrole ranging from 10–1000 µM, and the final concentration of DMSO being kept constant at 1%. Blank incubations were performed without the cofactor NADPH. Under these conditions the formation of the different metabolites of P450 mediated oxidation appeared to be linear with time and microsomal protein concentration (data not shown). Each experiment consisted of three replicates.

Determination of kinetic constants for glucuronidation of 1′-hydroxysafrole

Incubations with pooled male rat S9 protein (Sprague-Dawley) were performed to determine the kinetic constants (V_{max} and K_m) for glucuronidation of 1′-hydroxysafrole. The

incubation mixtures contained (final concentrations) 10 mM UDPGA, 0.2 mg S9 protein/mL, 0.2 M Tris-HCl (pH 7.4) and 10 mM $MgCl_2$. Preincubation of the mixture with 0.025 mg/mL alamethicin added from a 200 times concentrated stock in methanol during 15 min on ice was applied in order to obtain maximal glucuronidation activity (Fisher *et al.*, 2000). Preincubation at 37°C for 1 min was performed prior to addition of the substrate 1′-hydroxysafrole from a 200 times concentrated stock solution in DMSO. After 10 min at 37°C the reaction was terminated by adding 50 µL of cold acetonitrile to the total incubation volume of 200 µL. The samples were centrifuged for 5 min at 16000 g and frozen at -20°C until analysis by HPLC. Blank incubations were performed without the cofactor UDPGA. Three replicates of each experiment were performed. Formation of 1′-hydroxysafrole glucuronide was confirmed by performing additional incubations in which the samples obtained were treated with β-glucuronidase. For these incubations, 90 µL of the sample of a reaction mixture that has not been terminated by the addition of acetonitrile was added to 10 µL of 1M potassium phosphate (pH 6.2) and 2 µL of β-glucuronidase solution (0.3 units), followed by incubation for 1 h at 37°C.

Determination of kinetic constants for oxidation of 1′-hydroxysafrole

The kinetic constants for conversion of 1′-hydroxysafrole to 1′-oxosafrole were analyzed by incubations of pooled male rat liver microsomes (Sprague-Dawley) with concentrations of 1′-hydroxysafrole ranging from 10-4000 µM. The incubation mixtures contained (final concentrations) 3 mM NAD^+, 2 mM GSH, and 1 mg/mL microsomes in 0.2 M Tris HCl (pH 7.4). GSH was added as an agent to trap the formed 1′-oxosafrole and NAD^+ was used as a cofactor (Punt *et al.*, 2009). Preincubation at 37°C for 1 min was performed prior to addition of the substrate 1′-hydroxysafrole. After 10 min at 37°C the reaction was terminated by adding 25 µL of cold acetonitrile to the total incubation volume of 100 µL. The samples were centrifuged for 5 min at 16000 g and frozen at -20°C until analysis by HPLC. Three replicates of each experiment were performed.

Determination of kinetic constants for sulfation of 1′-hydroxysafrole

The sulfation of 1′-hydroxysafrole was performed using incubations of 1′-hydroxysafrole with male rat liver S9 protein of Sprague-Dawley in the presence of GSH as a scavenger of the reactive carbocation formed upon decomposition of the unstable 1′-sulfooxysafrole (Al-Subeihi *et al.*, 2011). The incubation mixtures of 100 µL contained (final concentrations) 10 mM GSH, 3 mg S9 protein/mL and 0.2 mM PAPS in 0.1 M potassium phosphate (pH 8.0). Preincubation at 37ºC for 1 min was performed prior to addition of 1′-hydroxysafrole from a 100 times concentrated stock solution in DMSO (final concentration 1% DMSO). The incubation was terminated after 2 h by addition of 25 µL cold acetonitrile. The samples were centrifuged for 5 min at 16000 g and frozen at -20°C until analysis by HPLC. Under these condition the formation of the glutathione adduct of the carbocation derived from 1′-sulfooxysafrole appeared to be linear with time and S9 protein concentration (data not shown). Determination of the kinetic constants for the formation of 1′-sulfooxysafrole from 1′-hydroxysafrole was performed with the same conditions as those described above, with the final concentration of 1′-hydroxysafrole ranging from 25-600 µM. Blank incubations were performed without GSH or male rat liver S9 protein. Three replicates of each experiment were performed.

Identification and quantification of metabolites of P450 mediated oxidation of safrole, and of 1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole

For the quantification of metabolites of P450 mediated oxidation of safrole, aliquots of 50 µL were analyzed on a Waters M600 HPLC equipped with photodiode array detector 2996 using an Alltima column C18 5 µm, 150 x 4.6 mm (Grace Alltech, Breda,The Netherlands). The gradient was made with ultrapure water and acetonitrile. The flow rate was 1 mL/min, starting at 100% ultrapure water and increasing to 15% acetonitrile in 5 min, after which the percentage of acetonitrile was increased to 30% and 38% in 20 min and 10 min respectively and kept at 38% for 10 min and then increased to 100% over 10 min and kept at this level for 5 min, after which the column was re-equilibrated in ultrapure water for 10 min. Quantification of 2′,3′-dihydroxysafrole and 1′-hydroxysafrole was achieved by comparison of the peak areas obtained at a wavelength of 286 nm to the calibration curve of

the corresponding synthesized reference compounds. The same method was used for the quantification of 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene at a wavelength of 266 nm and 280 nm respectively.

For the quantification of 1′-hydroxysafrole glucuronide, aliquots of 50 µL were analyzed on a Waters M600 HPLC equipped with a photodiode array detector 2996 using an Alltima column C18 5 µm, 150 x 4.6 mm (Grace Alltech, Breda, The Netherlands). The gradient was made with ultrapure water containing 0.1% (v/v) acetic acid and acetonitrile. The flow rate was 1 mL/min and a gradient was applied from 10% to 25% acetonitrile in 30 min, increased to 100% acetonitrile in 2 min and maintained at 100% acetonitrile for 1 min, after which acetonitrile was decreased to 10% within 2 min and maintained at this level for 10 min. Because 1′-hydroxysafrole glucuronide has the same UV spectrum as 1′-hydroxysafrole it was assumed that it has the same molar extinction coefficient as 1′-hydroxysafrole. Therefore, quantification was done by comparison of the peak area of 1′-hydroxysafrole glucuronide obtained at a wavelength of 286 nm to the calibration curve of the synthesized 1′-hydroxysafrole at the same wavelength.

For the quantification of 1′-oxosafrole, aliquots of 50 µL were analyzed on a Waters M600 HPLC equipped with photodiode array detector 2996 using an Alltima column C18 5 µm, 150 x 4.6 mm (Grace Alltech, Breda,The Netherlands). The mobile phase was made with ultrapure water containing 0.1% (v/v) acetic acid and acetonitrile. The flow rate was 1 mL/min and a gradient was applied from 0% to 25% acetonitrile over 5 min after which the percentage of acetonitrile was kept at 25% for 5 min and then increased to 100% over 3 min and kept at 100% for 3 min, after which the column was re-equilibrated in ultrapure water containing 0.1% (v/v) acetic acid for 10 min. Detection was performed at 312 nm. Quantification of 1′-oxosafrole was performed by means of a calibration curve obtained as described previously (Punt *et al.*, 2009). A calibration curve of 1′-oxosafrole was prepared by incubating 60 µM synthesized 1′-oxosafrole with increasing concentrations of GSH (0–20 µM) in 0.2 M Tris-HCl (pH 7.4). The reactions were incubated for 6 h at 37ºC resulting in the maximum formation of GS-1′-oxosafrole. The peak area of GS-1′-oxosafrole in the chromatograms of these reactions were related to the quantity of GSH used in the reactions,

thus providing the curve for the GSH adduct of 1′-oxosafrole ultimately quantifying the formation of 1′-oxosafrole.

For the quantification of 1′-sulfooxysafrole, aliquots of 3.5 µL were analyzed on a UPLC (Ultra Performance Liquid Chromatography)-DAD system consisting of a Waters (Milford, MA) Acquity binary solvent manager, sample manager and photodiode array detector, equipped with a Waters Acquity UPLC BEH RP 18 column (1.7 µm, 2.1 x 50 mm). The gradient eluent consisted of ultrapure water containing trifluoroacetic acid 0.1% (v/v) and acetonitrile and the flow rate was 0.6 mL/min. A gradient was applied from 20% to 30% acetonitrile over 4.5 min after which the percentage of acetonitrile was increased to 100% over 0.3 min and kept at 100% for 0.2 min. Thereafter, the percentage of acetonitrile was reduced to its initial value in 0.2 min and kept at this level for 1.5 min. Detection and quantification of 1′-sulfooxysafrole were performed at 266 nm. Because the UV-spectrum of the GSH adduct of 1′-sulfooxysafrole was similar to the UV-spectrum of 3′-hydroxysafrole, it was assumed that it has the same molar extinction coefficient as 3′-hydroxysafrole. Quantification of the GSH adduct of 1′-sulfooxysafrole was therefore achieved by comparison of the peak area of the GSH adduct of 1′-sulfooxysafrole in the chromatograms obtained at a wavelength of 266 nm to the calibration curve of 3′-hydroxysafrole at the same wavelength.

LC-MS analysis of 1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole

Formation of 1′-hydroxysafrole glucuronide in the incubations was verified by LC-MS which was performed on an Agilent 1200 HPLC system coupled to a Bruker MicroTOF mass spectrometer. Aliquots of 20 µL were separated on an Alltima C18 5 µm, 150 mm x 2.1 mm (Grace Alltech, Breda, The Netherlands). The gradient eluent was made with ultrapure water containing 0.1% (v/v) formic acid (eluent A) and acetonitrile with 0.1% (v/v) formic acid (eluent B) and the flow rate was set to 0.2 mL/min. A linear gradient was applied from 10% to 25% eluent B over 30 min after which the percentage of eluent B was increased to 100% over 2 min and kept at 100% for 1 min. Mass spectrometric analysis was in the negative electrospray mode using a spray capillary voltage of 4500 V, a capillary temperature of 200^0C and nitrogen as nebulizer gas at 8.0 L/min.

The nature of GS-1′-oxosafrole in the incubations was verified by LC-MS, which was performed on an Agilent 1200 HPLC system coupled to a Bruker MicroTOF mass spectrometer. Aliquots of 20 µL (injected volume) were separated on an Alltima C18 5 µm column, 150 x 2.1 mm (Grace Altech, Breda, The Netherlands). The gradient was made with acetonitrile (eluent B) and ultrapure water (eluent A) both containing 0.1% (v/v) formic acid and the flow rate was set to 0.2 mL/min. A linear gradient was applied from 0-25% eluent B over 5 min and was kept at 25% for 5 min, after which the percentage of eluent B was increased to 100% over 2 min. Mass spectrometric analysis was performed in positive electrospray mode using a spray capillary voltage of 4500 V, a capillary temperature of 200^0C and nitrogen as nebulizer gas at 8.0 L/min.

Formation of the glutathione conjugate of 1′-sulfooxysafrole was verified by LC-MS, which was performed on an Agilent 1200 HPLC system coupled to a Bruker MicroTOF mass spectrometer. Aliquots of 20 µL (injected volume) were separated on an Alltima C18 5 µm column, 150 x 2.1 mm (Grace Altech, Breda, The Netherlands). The gradient was made with ultrapure water containing 0.1% (v/v) formic acid (eluent A) and acetonitrile with 0.1% (v/v) formic acid (eluent B) and the flow rate was set to 0.2 mL/min. The percentage of eluent B was kept at 0% for 5 min after which a linear gradient was applied from 0% to 10% eluent B over 10 min and a subsequent increase to 30% in another 10 min after which the percentage of eluent B was increased to 80% over 10 min and kept at 80% for 2 min. 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.

Data analysis

The apparent maximum velocity ($V_{max(app)}$) and apparent Michaelis-Menten constant ($K_{m(app)}$) for the formation of the different P450 mediated oxidation products of safrole, and for glucuronidation, oxidation and sulfation of 1′-hydroxysafrole were determined by fitting the data to the standard Michaelis Menten equation: $v = V_{max*}[S]/(K_m+[S])$. In this equation, [S] represents the substrate concentration for P450 mediated oxidation reactions or 1′-hydroxysafrole for conjugation reactions. Fitting the data to Michaelis Menten equation

was done using GraphPad Prism version 5.03 (GraphPad Software, San Diego California U.S.). The values of V_{max} and K_m were reported in nmole per min per mg microsomal or S9 protein and µM respectively. Scaling of in vitro data, expressed as nmol/min/mg protein, to in vivo data, expressed as µmol/h, was performed as described below for the definition of the PBBK model.

Definition of the PBBK model for safrole metabolism

A PBBK model was developed to describe the dose dependent kinetic behavior of safrole and the formation of its metabolites in male rats. The model was based on a model previously defined for estragole (Punt *et al.*, 2008). A schematic presentation of the PBBK model used to simulate the metabolism and distribution of safrole in the rat is presented in Figure 2.2. The model consists of eight compartments including the liver, lung, and kidney as metabolizing compartments, and separate compartments for fat, arterial blood, venous blood, richly perfused and slowly perfused tissues. Safrole is assumed to be taken up from the gastrointestinal tract following first order kinetics and to be absorbed by the liver directly with an absorption constant set at 1.0 h^{-1}. This assumption was based on the fact that uptake of the related alkenylbenzene estragole from the gastrointestinal track is known to be rapid and complete with an absorption half-life of 0.7 h (Anthony *et al.*, 1987; Punt *et al.*, 2008). Metabolism of safrole resulting in the formation of 1′-hydroxysafrole and 3′-hydroxysafrole was observed in the liver, lung and kidney and therefore described for these organs whereas the formation of 2′,3′-dihydroxysafrole (derived from safrole 2′,3′-epoxide) and 1,2-dihydroxy-4-allylbenzene was observed and therefore modeled only in the liver compartment. The mass balance equations in the PBBK model for time dependent changes of safrole in the liver, kidney and lung were as follows:

Liver:
$$dAL_S/dt = dUptake_S/dt + QL*(CA_S - CL_S/PL_S) - V_{max,L\_DHS}*CL_S/PL_S/(K_{m,L\_DHS} + CL_S/PL_S) - V_{max,L\_1'HS}*CL_S/PL_S/(K_{m,L\_1'HS} + CL_S/PL_S) - V_{max,L\_3'HS}*CL_S/PL_S/(K_{m,L\_3'HS} + CL_S/PL_S)$$

$$-V_{max,L\_DHAB}*CL_S/PL_S/(K_{m,L\_DHAB}+CL_S/PL_S)$$

$$dUptake_S/dt = -dAGI_S/dt = K_a*AGI_S,\ AGI_S(0)= \text{oral dose}$$

$$CL_S = AL_S/VL$$

Kidney: $$dAK_S/dt = QK*(CA_S - CK_S/PK_S)$$

$$- V_{max,K\_3'HS}*CK_S/PK_S/(K_{m,K\_3'HS}+CK_S/PK_S)$$

$$CK_S = AK_S/VK$$

Lung: $$dALu_S/dt = QC*(CV_S - CLu_S/PLu_S)$$

$$-V_{max,K\_3'HS}*CLu_S/PLu_S/(K_{m,K\_3'HS}+CLu_S/PLu_S)$$

$$-V_{max,K\_1'HS}*CLu_S/PLu_S/(K_{m,K\_1'HS}+CLu_S/PLu_S)$$

$$CLu_S = ALu_S/VLu$$

where $uptake_S$ (in µmol) is the amount of safrole taken up from the gastrointestinal tract, AGI_S (in µmol) is the amount of safrole remaining in the gastrointestinal tract, ATi_S (in µmol) is the amount of safrole in a tissue (Ti= L (liver), K(kidney) or Lu (lung)). CTi_S (in µmol/L) is the safrole concentration in a tissue. CA_S and CV_S are the safrole concentrations in the arterial and venous blood (both in µmol/L), QTi (in L/h) is the blood flow rate to a tissue, QC (in L/h) is the cardiac output, VTi (in L) is the volume of a tissue, PTi_s is the tissue/blood partition coefficient of safrole and V_{max,Ti_M} and K_{m,Ti_M} are the values representing the apparent maximum rate and Michaelis-Menten constant for the formation of 2′,3′-dihydroxysafrole (DHS), 1′-hydroxysafrole (1′HS), 3′-hydroxysafrole (3′HS), and 1,2-dihydroxy-4-allylbenzene (DHAB). Formation of 2′,3′-dihydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene from safrole are taken into account but no further reaction of these metabolites were modeled. Formation of 1′-hydroxysafrole is taken into account in the liver and lung but its further conversion by conjugation reactions was only modeled in the liver and not in the lung because formation of this metabolite in

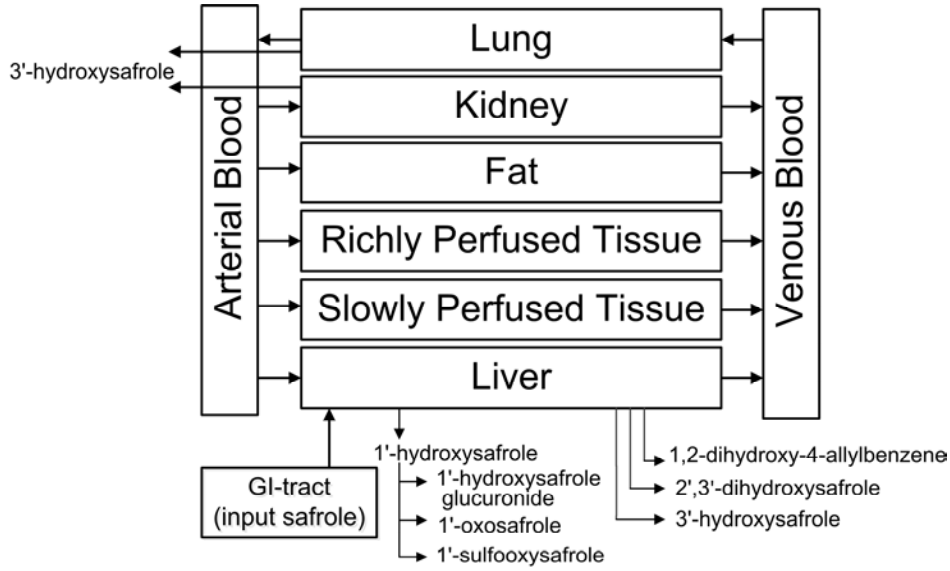

Figure 2.2. Schematic presentation of the physiologically based biokinetic (PBBK) model used to simulate the metabolism and distribution of safrole in rat.

the lung was considered too low to require further modeling of its conjugation reactions (see Results section). The mass balance equation for 1′-hydroxysafrole in the liver is as follows:

$$\text{Liver: } dAL_{1'HS}/dt = V_{max,L\_1'HS} * CL_S/PL_S/(K_{m,L\_1'HS} + CL_S/PL_S)$$

$$- V_{max,L\_1'HSG} * CL_{1'HS}/PL_{1'HS}/(K_{m,L\_1'HSG} + CL_{1'HS}/PL_{1'HS})$$

$$- V_{max,L\_1'HOS} * CL_{1'HS}/PL_{1'HS}/(K_{m,L\_1'HOS} + CL_{1'HS}/PL_{1'HS})$$

$$- V_{max,L\_1'HSS} * CL_{1'HS}/PL_{1'HS}/(K_{m,L\_1'HSS} + CL_{1'HS}/PL_{1'HS})$$

$$CL_{1'HS} = AL_{1'HS}/VL$$

where $AL_{1'HS}$ is the amount of 1′-hydroxysafrole in the liver (in µmol), $CL_{1'HS}$ is the 1′-hydroxysafrole concentration in the liver (in µmol/L), $PL_{1'HS}$ is the liver/blood partition coefficient of 1′-hydroxysafrole and V_{max,L_M} and K_{m,L_M} are the apparent maximum rate and

Michaelis-Menten constant for the formation in the liver of the various conjugated metabolites, including 1′-hydroxysafrole glucuronide (1′HSG), 1′-oxosafrole (1′HOS) and 1′-sulfooxysafrole (1′HSS).

The metabolites 2′,3′-dihydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene formed in all organs and 1′-hydroxysafrole glucuronide and 1′-oxosafrole formed in the liver were all assumed to be completely excreted in the urine. Transport of metabolites from the liver to the kidney and urine was not included in the model. PBBK model predictions of the dose dependent formation of the various P450 mediated oxidation products of safrole and of the various conjugated metabolites were performed over 72 h.

The kinetic parameters for the formation of P450 mediated oxidation products of safrole and for the glucuronidation, oxidation and sulfation of 1′-hydroxysafrole were determined in vitro. The V_{max} values for the formation of P450 mediated oxidation products and conjugated metabolites obtained from incubations with microsomal liver samples were scaled to the liver using a microsomal protein yield of 35 mg/g liver (Medinsky *et al.*, 1994; Punt *et al.*, 2008). The V_{max} values for metabolites formed in the lung and kidney were scaled using a microsomal protein yield of 20 mg/g lung and 7 mg/g kidney (Beierschmitt and Weiner, 1986; Medinsky *et al.*, 1994; Punt *et al.*, 2008). The V_{max} values for sulfation and glucuronidation of 1′-hydroxysafrole were scaled to the liver using an S9 protein yield of 143 mg/g liver.

The physiological parameter values including organ volumes, cardiac output, and blood flows were taken from literature (Brown *et al.*, 1997) and are shown in Table 2.1. Partition coefficients were estimated from the log K_{ow} based on the method described previously (DeJongh *et al.*, 1997). The values of *C* log *P* for safrole and 1′-hydroxysafrole were calculated with ChemBio3D Ultra 2010 (CambridgeSoft, U.S.) and amounted to 3.18 and 1.62. Mass balance and differential equations of safrole metabolism were coded and simulated with Berkeley Madonna 8.3.14 (Macey and Oster, UC Berkeley, CA, U.S.) using the Rosenbrock′s algorithm for stiff system.

Sensitivity analysis

Sensitivity analysis was used to identify the parameters that have the greatest impact on a specified model output (DeWoskin, 2007). Normalized sensitivity coefficients (*SC*) were determined according to the following equation: $SC = (C' - C)/(P' - P)*(P/C)$, 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' is the modified parameter value (Evans and Andersen, 2000; Garg and Balthasar, 2007; Tardif *et al.*, 2002). On the basis of literature data (Punt *et al.*, 2008), an increase of 5% in parameter values was chosen to analyze the effect of a change in parameter on the formation of 1,2-dihydroxy-4-allylbenzene, 1′-hydroxysafrole, 1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole (expressed as % of dose over 72 h). The final coefficients were calculated using Excel (Excel 2007, Microsoft Corporation, WA).

Table 2.1. Parameters used in the physiologically based biokinetic model for safrole in rat

Physiological parameters[a]		Tissue: blood partition coefficients[b]	
body weight (kg)	0.25		
percentage of body weight		safrole	
liver	3.4	liver	2.50
lungs	0.5	lung	2.50
kidney	0.7	kidney	2.50
fat	7.0	fat	80.75
rapidly perfused	4.4	rapidly perfused	2.50
slowly perfused	67.6	slowly perfused	0.82
arterial blood	1.85	1′-hydroxysafrole	
venous blood	5.55	liver	1.13
cardiac output (L/h/kg $bw^{0.74}$)	15.0		
percentage of cardiac output			
liver	25.0		
kidney	14.1		
fat	7.0		
rapidly perfused	36.9		
slowly perfused	17.0		

[a] Brown *et al.* (1997)

[b] DeJongh *et al.* (1997)

Comparison of PBBK model based predictions for safrole to the PBBK model based predictions for the related alkenylbenzenes estragole and methyleugenol

The PBBK model based predictions obtained for safrole were compared to those obtained with previously developed PBBK models for the related alkenylbenzenes estragole and methyleugenol (Al-Subeihi *et al.*, 2011; Punt *et al.*, 2008). To this end, the three PBBK models were run to obtain i) the predicted time dependent concentration of alkenylbenzenes in the liver and ii) the dose dependent formation of the respective 1′-hydroxy metabolites, the 1′-hydroxy glucuronide metabolites, the 1′-oxo metabolites, and the 1′-sulfooxy metabolites of estragole, methyleugenol, and safrole. For the dose dependent prediction of metabolite formation the PBBK models were run from dose levels of 0.0001 mg/kg bw up to 1000 mg/kg bw, and for 24 h in the case of estragole and methyleugenol and 72 h in the case of safrole.

Results

P450 mediated oxidation of safrole

HPLC analysis of incubations with rat liver, lung, kidney and small intestine microsomes in the presence of safrole as substrate and NADPH as cofactor reveals that P450 mediated conversion of safrole only occurs in incubations with rat liver, lung and kidney (Figure 2.3), whereas no conversion of safrole was found in incubations with male rat small intestinal microsomes (data not shown). The metabolites formed in incubations with rat liver microsomes are 2′,3′-dihydroxysafrole, 1′-hydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene (Figure 2.3A). The HPLC chromatograms of incubations of rat lung microsomes revealed the formation of 3′- hydroxysafrole and 1′-hydroxysafrole (Figure 2.3B) and those of kidney microsomes the formation of 3′-hydroxysafrole (Figure 2.3C). These metabolites were identified on the basis of the similarity of their UV spectrum and retention time compared to the UV spectrum and retention time of synthesized or commercially available reference compounds. Under the conditions applied the retention time for 2′,3′-dihydroxysafrole, 1′-hydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene were 17, 28, 29, and 30 min respectively. In these microsomal incubations, safrole 2′,3′-oxide was not detected because of its swift hydrolysis by microsomal epoxide

hydrolase to 2′,3′-dihydroxysafrole (Delaforge *et al.*, 1980; Guenthner and Luo, 2001; Luo *et al.*, 1992; Stillwell *et al.*, 1974).Formation of 3′-hydroxysafrole was shown to result directly from safrole metabolism and not from the isomerization of 1′-hydroxysafrole since incubation of 1′-hydroxysafrole with liver microsomes and the cofactor NADPH did not result in the formation of 3′-hydroxysafrole (data not shown).

The formation of 1′-hydroxysafrole in incubations with liver microsomes is higher than that in incubations with lung microsomes while in the incubations with kidney microsomes no formation of 1′-hydroxysafrole was observed. The data indicate that extrahepatic P450 mediated oxidation contributes to a minor extent to the overall P450 mediated oxidation of safrole.

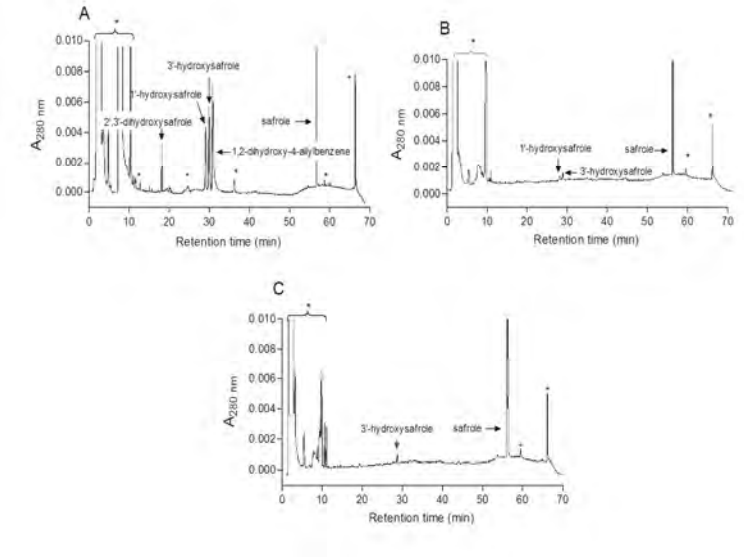

Figure 2.3. HPLC chromatograms of incubations of safrole with male rat (A) liver, (B) lung, and (C) kidney microsomes at a substrate concentration of 1000 µM and NADPH as a cofactor. The peaks marked with an asterisk were also present in the blank incubations without NADPH.

Kinetics of P450 mediated oxidation of safrole

Figure 2.4A shows the rate of formation of 2′,3′-dihydroxysafrole, 1′-hydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene in incubations with male rat liver

microsomes and increasing concentrations of safrole (10-1000 µm). Similar experiments were performed for lung and kidney microsomes (500-4000 µM) (Figure 2.4B and 2.4C). Table 2.2 presents the apparent K_m and V_{max} values obtained from these plots and the catalytic efficiencies (V_{max}/K_m) derived from them.

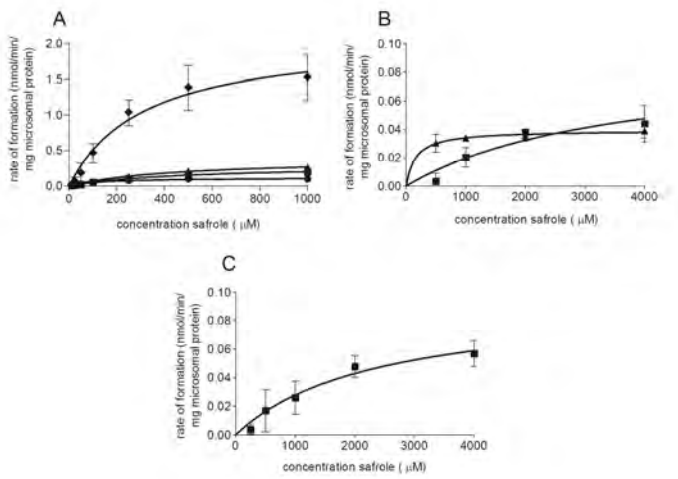

Figure 2.4. Safrole concentration dependent rate of formation of P450 metabolites in incubations with rat (A) liver, (B) lung, and (C) kidney microsomes and NADPH as a cofactor. In the plots each point represents the mean (± SD) of three replicates corresponding to the formation of 2′,3′-dihydroxysafrole (•), 1′-hydroxysafrole (▲), 3′-hydroxysafrole (■) and 1,2-dihydroxy-4-allylbenzene (◆).

The results obtained reveal that with male rat liver microsomes 1,2-dihydroxy-4-allylbenzene is the major metabolite formed, followed by 1′-hydroxysafrole, 3′-hydroxysafrole and 2′,3′-dihydroxysafrole. The catalytic efficiency for the formation of 1,2-dihydroxy-4-allylbenzene, resulting from O-dealkylation is about 8 fold higher than that for the formation of 1′-hydroxysafrole.

The results obtained with male rat lung microsomes reveal that 1′-hydroxysafrole is the most abundant metabolite formed in the lung (Figure 2.4B). In the lung, the apparent V_{max} for the formation of 1′-hydroxysafrole is about 2.5 times lower than the apparent V_{max} for

the formation of 3′-hydroxysafrole whereas the apparent K_m is about 22 times lower. Overall, the catalytic efficiency for the formation of 1′-hydroxysafrole by male rat lung microsomes is about 8 times higher than that the catalytic efficiency for the formation of 3′-hydroxysafrole.

The results obtained with male rat kidney microsomes reveal that only 3′-hydroxysafrole is formed (Figure 2.4C). The apparent V_{max}, K_m and catalytic efficiency calculated as the apparent V_{max}/K_m of 3′-hydroxysafrole were 3067 µM, 0.1 nmol/ min/mg microsomal protein and 0.03 µL/min/mg microsomal protein respectively.

The relatively large variations in the kinetic values for formation of 3′-hydroxysafrole are mainly caused by the fact that 3′-hydroxysafrole was also formed in the blank incubations without co-factor NADPH. Correcting the amount of 3′-hydroxysafrole formed in full incubations for the amount formed in the blank, contributed to the experimental variation.

Table 2.2. The kinetic parameters for P450 mediated oxidation of safrole in incubations with liver, lung and kidney microsomes from male Sprague-Dawley rats

Organ	Metabolite	$K_{m\ (app)}$ (µM)[a]	$V_{max\ (app)}$ (nmol/min/mg microsomal protein)[a]	In vitro catalytic efficiency ($V_{max(app)}/K_{m(app)}$) (µL/min/mg microsomal protein)	Scaled $V_{max,\ in\ vivo}$ (µmol/h)	In vivo catalytic efficiency (scaled $V_{max,\ in\ vivo}/K_{m\ (app)}$) (L/h)
Liver	2′,3′-dihydroxysafrole	149 ± 46	0.12±0.02	0.81	2.14[b]	0.014
	1′-hydroxysafrole	497 ± 190	0.40 ± 0.09	0.81	7.2 [b]	0.015
	3′-hydroxysafrole	546 ± 370	0.33 ±0.17	0.61	5.9 [b]	0.011
	1,2-dihydroxy-4-allylbenzene	313 ±27	2.1 ±0.49	6.7	37.5[b]	0.12
Lung	1′-hydroxysafrole	159 ±66	0.04 ±0.003	0.25	0.06[c]	4.10^{-4}
	3′-hydroxysafrole	3573 ±2152	0.1 ± 0.03	0.03	0.15[c]	4.10^{-5}
Kidney	3′-hydroxysafrole	3067±1966	0.1 ± 0.015	0.03	0.07[d]	2.10^{-5}

[a] The values are mean ± SD of three independent determinations
[b] $V_{max\ (app)}$/(1000 nmol/µmol)*(60 min/h)*(35 mg microsomal protein/g liver)*(34 g liver/kg bw)*(0.25 kg bw)
[c] $V_{max\ (app)}$/(1000 nmol/µmol)*(60 min/h)*(20 mg microsomal protein/g lung)*(5 g lung/kg bw)*(0.25 kg bw)
[d] $V_{max\ (app)}$/(1000 nmol/µmol)*(60 min/h)*(7 mg microsomal protein/g kidney)*(7 g kidney/kg bw)*(0.25 kg bw)

Comparing the in vivo catalytic efficiencies for the formation of P450 mediated metabolites in different organs shows that in the male rat conversion of safrole is most efficiently catalyzed in the liver with 1,2-dihydroxy-4-allylbenzene as the most abundant metabolite. The in vivo catalytic efficiency for the formation of the proximate carcinogenic metabolite 1′-hydroxysafrole is approximately 9% of the total catalytic efficiency of the liver.

Glucuronidation of 1′-hydroxysafrole

An HPLC chromatogram of an incubation of male rat liver S9 with 1′-hydroxysafrole as the substrate and UDPGA as cofactor to study glucuronidation of 1′-hydroxysafrole is presented in Figure 2.5A. Formation of a single metabolite eluting at a retention time of 24 min was observed. Treatment of the sample with β-glucuronidase resulted in complete elimination of this metabolite and a concomitant increase in the intensity of the peak of 1′-hydroxysafrole, revealing that the metabolite formed corresponds to the glucuronosyl conjugate of 1′-hydroxysafrole.

Furthermore, the spectrum of the metabolite obtained by LC-MS reveals a deprotonated molecule at *m/z* 353, which corresponds to the theoretically expected mass and confirms that the metabolite corresponds to 1′-hydroxysafrole glucuronide.

Kinetics of glucuronidation of 1′-hydroxysafrole

Figure 2.5B shows the rate of glucuronidation of 1′-hydroxysafrole with a substrate concentration increasing from 5-1000 µM. Kinetic constants obtained from this graph included an apparent V_{max} amounting to 25 nmol/min/mg S9 protein and an apparent K_m value of 101 µM. The catalytic efficiency for glucuronidation of 1′-hydroxysafrole (V_{max}/K_m) is 248 µL/min/mg S9 protein (Table 2.3).

Oxidation of 1′-hydroxysafrole

Figure 2.6A shows the HPLC chromatogram of an incubation of 1′-hydroxysafrole with male rat liver microsomes and NAD^+ as cofactor and GSH as a trapping agent. Formation of a single metabolite with a retention time of 12 min was observed. On the basis of LC-MS analysis, the metabolite could be identified as 1′-GS-oxosafrole because the spectrum

reveals a deprotonated molecule at *m/z* 484 which corresponds to the theoretically expected mass.

Kinetics of oxidation of 1′-hydroxysafrole

Figure 2.6B shows the rate of oxidation of 1′-hydroxysafrole with increasing substrate concentrations ranging from 10-4000 µM. The data obtained revealed an apparent V_{max} of 5.9 nmol/min/mg microsomal protein and an apparent K_m of 1809 µM.

Comparison of the kinetic constants for the oxidation of 1′-hydroxysafrole with those for the glucuronidation of 1′-hydroxysafrole that were scaled to the in vivo situation (Table 2.3) reveals that the catalytic efficiency for the formation of 1′-oxosafrole was about 300 times lower than those for the formation of 1′-hydroxysafrole glucuronide.

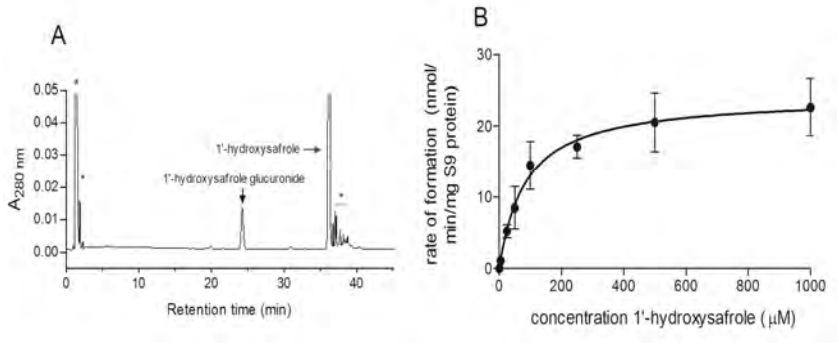

Figure 2.5 HPLC chromatogram of an incubation of 1′-hydroxysafrole (1 mM) with male rat liver S9 in the presence of UDPGA as a cofactor (A) and 1′-hydroxysafrole concentration dependent rate of formation of 1′-hydroxysafrole glucuronide (B). In the plot each point represents the mean (± SD) of three replicates.

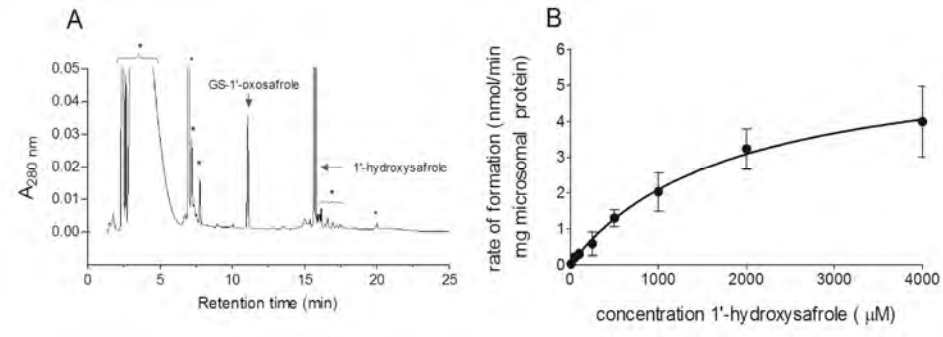

Figure 2.6. HPLC chromatogram of an incubation of 1′-hydroxysafrole (1 mM) with male rat liver microsomes in the presence of NAD^+ as a cofactor and GSH as a trapping agent (A) and 1′-hydroxysafrole concentration dependent rate of formation of 1′-oxosafrole (B). In the plot each point represents the mean (± SD) of three replicates.

Table 2.3. Kinetic parameters for conjugation reactions of 1′-hydroxysafrole in incubations with liver microsomes and S9 protein from male Sprague-Dawley rats

Organ	Metabolite	$K_{m\ (app)}$ (μM)[a]	$V_{max\ (app)}$ (nmol/min/mg microsomal or S9 protein)[a]	In vitro catalytic efficiency ($V_{max\ (app)}/K_{m\ (app)}$) (μL/min/mg microsomal or S9 protein)	Scaled $V_{max,\ invivo}$ (μmol/h)	In vivo catalytic efficiency (scaled $V_{max,\ in\ vivo}/K_{m\ (app)}$) (L/h)
Liver	1′-hydroxysafrole glucuronide	101±62	25±5.7	248	1823[b]	18
	1′-oxosafrole	1809±87	5.9±1.4	3.2	105[c]	0.06
	1′-sulfooxysafrole	127±19	0.03± 0.002	0.24	2.12[b]	0.017

[a] The values are mean ± SD of three independent determinations
[b] $V_{max\ (app)}$ /(1000 μmol/nmol)*(60 min/h)*(143 mg S9 protein/g liver)*(34 g liver/kg bw)*(0.25 kg bw)
[c] $V_{max\ (app)}$ /(1000 μmol/nmol)*(60 min/h)*(35 mg microsomal protein/g liver)*(34 g liver/kg bw)*(0.25 kg bw)

Sulfation of 1′-hydroxysafrole

Figure 2.7A shows the UPLC chromatogram of an incubation of 1′-hydroxysafrole with male rat liver S9 using PAPS as cofactor and GSH as trapping agent for the reactive carbocation derived from the unstable 1′-sulfooxysafrole metabolite. Formation of a single

metabolite with a retention time of 1.23 min was observed. HPLC analysis of incubations of 1′-sulfooxysafrole in the absence of GSH and in the presence of S9 protein and PAPS revealed no peak formation at 1.23 min (chromatogram data not shown). This argument was used to confirm the formation of the GSH adduct of the carbocation of 1′-hydroxysafrole and thus an indirect detection of the formation of 1′-sulfooxysafrole in the complete incubation.

Furthermore, the spectrum of the metabolite obtained by LC-MS analysis of this metabolite reveals a deprotonated molecule at *m/z* 468 which corresponds to the theoretically expected mass and confirms that the metabolite corresponds to the GSH adduct of the carbocation formed from 1′-sulfooxysafrole.

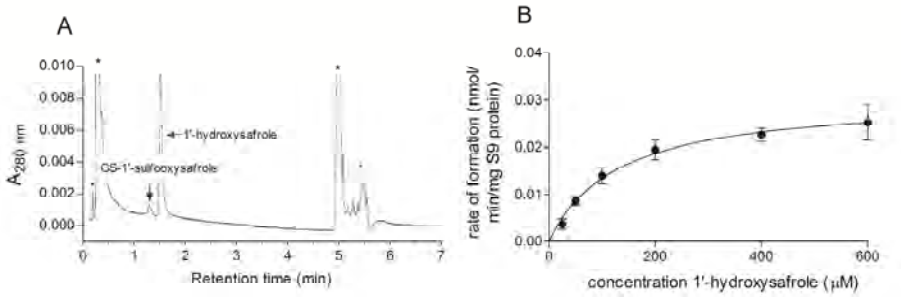

Figure 2.7. Figure 2.7. UPLC chromatogram of an incubation of 1′-hydroxysafrole (1 mM) with male rat liver S9 homogenates in the presence of PAPS as a cofactor and GSH as a trapping agent (A) and 1′-hydroxysafrole concentration dependent rate of formation of 1′-sulfooxysafrole (B). In the plot each point represents the mean (± SD) of three replicates.

Kinetics of sulfation of 1′-hydroxysafrole

Figure 2.7B shows the rate of sulfation of 1′-hydroxysafrole with a substrate concentration increasing from 25-600 µM. The data obtained revealed an apparent V_{max} of 0.03 nmol/min/mg S9 protein and an apparent K_m of 127 µM. The catalytic efficiency for the sulfation of 1′-hydroxysafrole (V_{max}/K_m) is 0.24 µL/min/mg S9 protein.

Comparison of the kinetic constants for sulfation of 1′-hydroxysafrole with those for glucuronidation and oxidation of 1′-hydroxysafrole that were scaled to the in vivo situation (Table 2.3) reveals that the catalytic efficiency for formation of 1′-sulfooxysafrole was about 1058 times and 3.5 times lower than those for the formation of 1′-hydroxysafrole glucuronide and 1′-oxosafrole respectively.

Altogether, it can be concluded that in the male rat liver, glucuronidation is the major pathway for the conversion of 1′-hydroxysafrole followed by oxidation and sulfation of 1′-hydroxysafrole to 1′-sulfooxysafrole is a minor pathway.

Performance of the PBBK model

The PBBK model defined when incorporating all of the kinetic constants and parameters defined (Tables 2.1, 2.2 and 2.3) was used to predict time and dose dependent safrole metabolism at low and high dose levels. To this end dose levels of 0.05 and 300 mg/kg bw were chosen to allow for the comparison with the outcomes obtained previously using the PBBK model for estragole (Punt *et al.*, 2008).

The PBBK model predicts that at doses ranging from 0.05 to 300 mg/kg bw about 99% of the metabolism of safrole takes place in the liver. The major metabolites of safrole formed in the liver are 1,2-dihydroxy-4-allylbenzene and 1′-hydroxysafrole which were formed for respectively 100% and 99% in the liver amounting to ~74% and ~10% of the dose within the dose-range of 0.05-300 mg/kg bw. Extrahepatic conversion of safrole did not contribute significantly to the overall formation of these metabolites.

Performance of the constructed PBBK model was evaluated by comparison of the predicted total metabolite formation to the reported total urinary excretion of safrole metabolites as a percentage of the dose in male Sprague-Dawley rats exposed to different levels of safrole (Benedetti *et al.*, 1977) and by comparison of the percentage of the dose predicted to be excreted as 1′-hydroxysafrole glucuronide and 1,2-dihydroxy-4-allylbenzene in the urine as compared to the actual reported level of excretion of these metabolites in the urine of Wistar rats upon intragastrical administration of safrole at a dose level of 162 mg/kg bw (Klungsøyr and Scheline, 1983).

Figure 2.8 shows the comparison between the PBBK predicted and the reported experimental data on the level of total safrole metabolites excreted in urine at different oral dose levels of safrole. At a dose level of 0.63 mg/kg bw, the PBBK model-predicted percentage of the dose excreted as metabolites and the actual percentage excreted in an in vivo rat experiment (Benedetti *et al.*, 1977) are similar (Figure 2.8A). At 6 h postdosing, the PBBK model predicted the percentage of the dose excreted as safrole metabolites to amount to 51% and the experimental value reported in the literature (Benedetti *et al.*, 1977) amounted to 45%, whereas at 12 hours these values amount to respectively 67 and 78% of the dose. At a dose of 60 mg/kg bw (Figure 2.8B) only limited experimental data are available, but the three data points reported amount to values of 18%, 78% and 85% of the dose, values that match the predicted values relatively well since these amount to 46%, 84% and 97% of the dose. At a dose of 745 mg/kg bw, (Figure 2.8C) deviation between the PBBK predicted levels of excretion and the actually observed levels of excretion are somewhat larger especially in the first hour after dosing where the predicted levels are 6.5 fold higher than the measured levels, although at 24 h or more upon dosing the predicted levels again match the experimental levels well and amount to values that were only 1.1-2.3 fold higher than the reported levels.

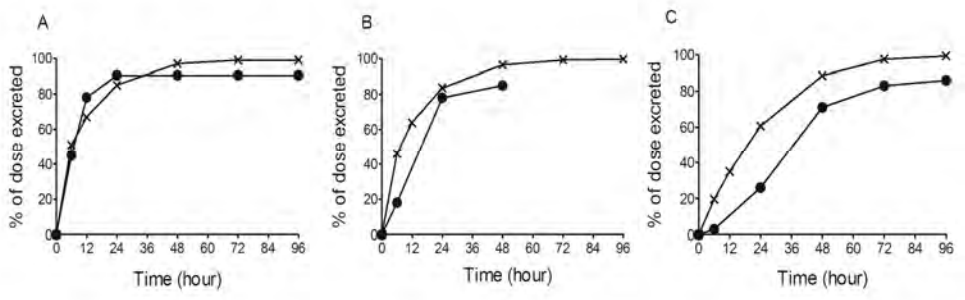

Figure 2.8. PBBK predicted (x) and reported (•) (Benedetti *et al.*, 1977) percentage of the safrole dose excreted as urinary metabolites at oral dose levels of (A) 0.63 mg/kg bw (B) 60 mg/kg bw, and (C) 745 mg/kg bw.

The model was also evaluated by comparing the PBBK model based prediction for the percentage of the dose excreted as 1′-hydroxysafrole glucuronide and 1,2-dihydroxy-4-allylbenzene to the values reported in a study in which Wistar rats were exposed to 162 mg/kg bw safrole (Klungsøyr and Scheline, 1983). Figure 2. 9 shows that the model accurately predicts the percentage of 1,2-dihydroxy-4-allylbenzene excreted in urine. The predicted formation of 1,2-dihydroxy-4-allylbenzene 24, 48 and 72 h after oral dosing was 60%, 72% and 74% of the dose, respectively, whereas the levels actually measured amounted to 58%, 70% and 72% respectively. The PBBK model prediction for the formation of 1′-hydroxysafrole glucuronide 24, 48 and 72 h upon dosing are 2-2.5 times higher than reported levels but within the same order of magnitude (Figure 2.9). Predicted levels of 1′-hydroxysafrole glucuronide amounted to 7.9%, 9.3% and 9.6% of the dose respectively whereas the reported levels amounted to 3.9%, 4.0% and 4.0%.

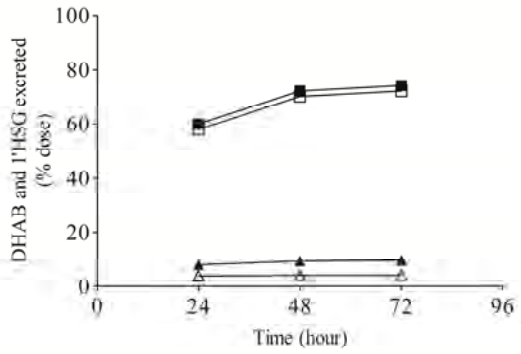

Figure 2.9. PBBK predicted formation of 1,2-dihydroxy-4-allylbenzene (■), and 1′-hydroxysafrole glucuronide (▲) compared to experimental data reported for formation of 1,2-dihydroxy-4-allylbenzene (□), and 1′-hydroxysafrole glucuronide (△) in rats exposed to 162 mg/kg bw safrole (Klungsøyr and Scheline, 1983).

Model predictions

In a next step, the PBBK model was used to predict the concentration of safrole and 1′-hydroxysafrole and the further metabolism of 1′-hydroxysafrole to 1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole in the liver. The PBBK model-predicted time dependent concentration of safrole and 1′-hydroxysafrole and the formation of 1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole in male rat liver upon exposure to 0.05 or 300 mg/kg bw of safrole are presented in Figure 2.10. At a dose 0.05 mg/kg bw the maximum concentration of safrole and 1′-hydroxysafrole were predicted to be achieved within less than 1 h while full conversion of safrole and maximal overall formation of 1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole were predicted to be achieved within 48 h. The maximum concentrations of safrole and 1′-hydroxysafrole and the overall formation of 1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole are 0.23, 0.00008, 0.8, 0.0026 and 0.0007 nmol/g liver respectively. Formation of 1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole corresponds to 8.9%, 0.03% and 0.008% of the dose respectively.

PBBK model predictions at a dose of 300 mg/kg bw reveal that safrole and 1′-hydroxysafrole were metabolized completely after 72 h and that the maximum concentrations of safrole and 1′-hydroxysafrole were obtained within 1 h with values of 1700 and 0.256 nmol/g liver, respectively (Figure 2.10B). The formation of 1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole was predicted to amount to 5370, 17.4 and 5.1 nmol/g liver which corresponds to 9.9%, 0.03% and 0.009% of the dose, respectively. These percentages were similar to the percentages of formation predicted at a dose level of 0.05 mg/kg bw.

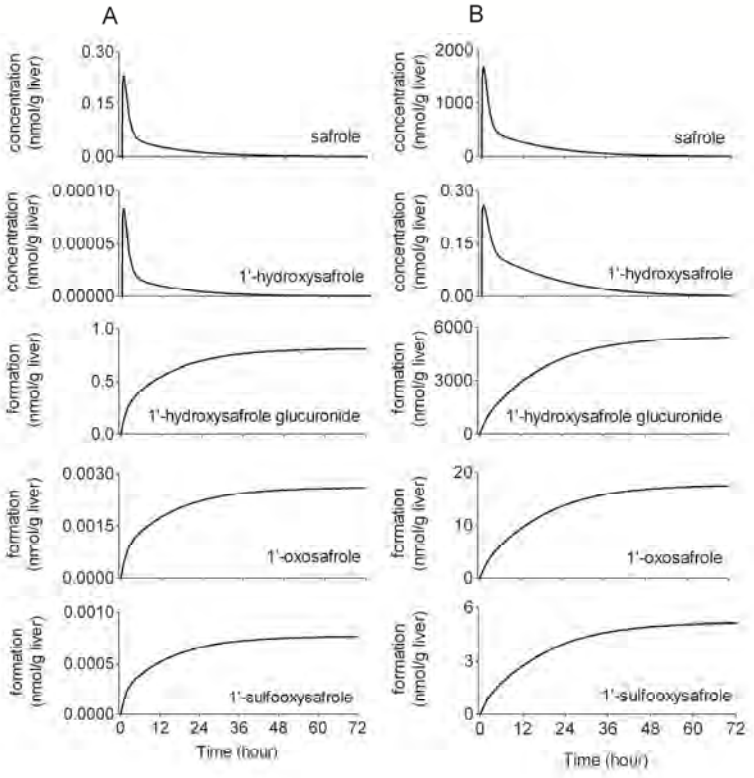

Figure 2.10. PBBK model predicted time dependent concentration (nmol/g liver) of safrole and 1′-hydroxysafrole and formation of 1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole at a dose level of (A) 0.05 mg/kg bw safrole and (B) 300 mg/kg bw safrole.

The PBBK model based predictions for the percentage of safrole metabolized to the various P450 metabolites at increasing oral doses of safrole are presented in Figure 2.11. At a dose of 0.05 mg/kg bw the percentage of safrole metabolized to 2′,3′-dihydroxysafrole, 1′-hydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene was, respectively, 8.9%, 9.1%, 6.7%, and 74.5% of the dose. When the dose was increased to 300 mg/kg bw, approximately 6.9%, 10.0%, 7.7% and 74.0% of the dose was predicted to be metabolized to 2′,3′-dihydroxysafrole, 1′-hydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-

allylbenzene respectively. These results reveal that 1,2-dihydroxy-4-allylbenzene is the major metabolite at low but also at high dose levels. When the dose increases the relative formation of 2′,3′-dihydroxysafrole decreased only to a limited extent from 8.9% at a dose 0.05 mg/kg bw to approximately 6.9% of the dose at a dose 300 mg/kg bw while the percentage formation of 1′-hydroxysafrole and 3′-hydroxysafrole tended to increase also only to a limited extent from values amounting to, respectively, 9.1% and 6.7% of the dose at 0.05 mg/kg bw to values of, respectively, 10.0% and 7.7% of the dose at 300 mg/kg bw/day. Overall, the results presented in Figure 2.11 reveal that for safrole a dose dependent shift in P450 mediated oxidation leading to a relative increase in bioactivation at higher dose levels, is not observed. This can be explained by the fact that for P450 mediated oxidation of safrole no saturation of an important metabolic route occurs in a dose range from 0.05 up to 300 mg/kg bw/day.

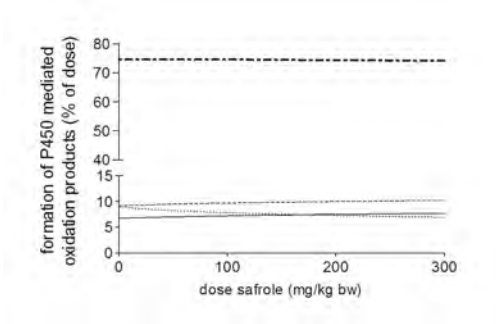

Figure 2.11. PBBK predicted dose dependent changes in overall formation of P450 metabolites in all organs. The lines correspond to 2′,3′-dihydroxysafrole (••••), 1′-hydroxysafrole (— —), 3′-hydroxysafrole (——), and 1,2-dihydroxy-4-allylbenzene (— • —).

Sensitivity analysis

A sensitivity analysis was performed to identify the key parameters that influence the formation of 1,2-dihydroxy-4-allylbenzene, 1′-hydroxysafrole, 1′ hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole.

Figure 2.12A presents that at both dose levels analyzed, 0.05 and 300 mg/kg bw, the formation of 1,2-dihydroxy-4-allylbenzene is influenced by the kinetic constants for its formation from safrole (V_{max,L_DHAB} and K_{m,L_DHAB}).

Figure 2.12B presents the sensitivity analysis for 1′-hydroxysafrole formation. Formation of 1′-hydroxysafrole is predicted to be mostly affected by the kinetic constants for its formation from safrole ($V_{max,L\_1'HS}$ and $K_{m,L\_1'HS}$) and kinetic constants for the formation of 1,2-dihydroxy-4-allylbenzene (V_{max,L_DHAB} and K_{m,L_DHAB}).

Figure 2.12C shows the results of the sensitivity analysis on the kinetic parameters which influence the formation of 1′-hydroxysafrole glucuronide. Glucuronidation of 1′-hydroxysafrole was affected by the kinetic constants for formation of 1′-hydroxysafrole ($V_{max,L\_1'HS}$, $K_{m,L\_1'HS}$) and the kinetic constants for formation of 1,2-dihydroxy-4-allylbenzene (V_{max,L_DHAB} and K_{m,L_DHAB}).

Figure 2.12D reveals that oxidation of 1′-hydroxysafrole was affected by the kinetic constants for formation of 1′-hydroxysafrole ($V_{max,L\_1'HS}$ and $K_{m,L\_1'HS}$), for glucuronidation of 1′-hydroxysafrole ($V_{max,L\_1'HSG}$ and $K_{m,L\_1'HSG}$), for oxidation of 1′-hydroxysafrole ($V_{max,L\_1'HOS}$ and $K_{m,L\_1'HOS}$), the kinetic constants for formation of 1,2-dihydroxy-4-allylbenzene (V_{max,L_DHAB} and K_{m,L_DHAB}), the yield of microsomal liver protein (MPL), and the yield of S9 protein (S9PL).

Figure 2.12E reveals that sulfation of 1′-hydroxysafrole was affected by the kinetic constants for the formation of 1′-hydroxysafrole ($V_{max,L\_1'HS}$ and $K_{m,L\_1'HS}$), the kinetic constants for the formation of 1,2-dihydroxy-4-allylbenzene (V_{max,L_DHAB} and K_{m,L_DHAB}), for the glucuronidation of 1′-hydroxysafrole ($V_{max,L\_1'HSG}$ and $K_{m,L\_1'HSG}$), and for the sulfation of 1′-hydroxysafrole ($V_{max,L\_1'HSS}$ and $K_{m,L\_1'HSS}$).

Comparison of the PBBK model based predictions for safrole to the PBBK model based predictions for the related alkenylbenzenes estragole and methyleugenol

Figure 2.13A shows that at a dose of 0.05 mg/kg bw, safrole was metabolized within 48 h which is longer than the 5 and 15 h predicted previously by the related PBBK models for estragole and methyleugenol respectively (Al-Subeihi *et al.*, 2011; Punt *et al.*, 2008). At a

dose of 300 mg/kg bw, safrole was predicted to be metabolized within 72 h whereas the corresponding values for estragole and methyleugenol amounted to respectively 15 and 20 h (Figure 2.13B).

Using these PBBK models for predicting bioactivation and detoxification of estragole, methyleugenol and safrole the relative time and dose dependent formation of the proximate carcinogenic 1′-hydroxy metabolites and also of the 1′-hydroxy glucuronide and the ultimate carcinogenic reactive and unstable 1′-sulfooxy metabolites of the three model alkenylbenzenes can be obtained. Figure 2.14 presents the formation of these metabolites for the three alkenylbenzenes as predicted by the respective PBBK models. Figure 2.14A and 14B show that formation of the 1′-hydroxy metabolite and 1′-hydroxy glucuronide for the three alkenylbenzenes are very similar. Figure 2.14C shows that the formation of 1′-oxomethyleugenol is predicted to be approximately less than 20-fold higher than the formation of 1′-oxoestragole and 1′-oxosafrole. Figure 2.14D reveals that the formation of the unstable reactive 1′-sulfooxy metabolite in the liver of male rat is predicted to vary less than 6-fold for the three alkenylbenzenes, being highest for methyleugenol, mainly because of additional differences in the catalytic efficiency of the conversion of the 1′-hydroxy metabolites in the various conjugation reactions. The data obtained also reveal that for all three alkenylbenzenes the formation of the ultimate carcinogenic 1′-sulfooxy metabolite increases linearly with the dose up to dose levels of at least 100 mg/kg bw and that the overall conversion of safrole is slower than that of estragole and methyleugenol taking about 72 instead of 24 h for full conversion. This can be explained by the fact that for P450 mediated oxidation of safrole, in contrast to that of estragole and methyleugenol, no saturation of an important metabolic route occurs in a dose range from 0.05 up to 300 mg/kg bw/day.

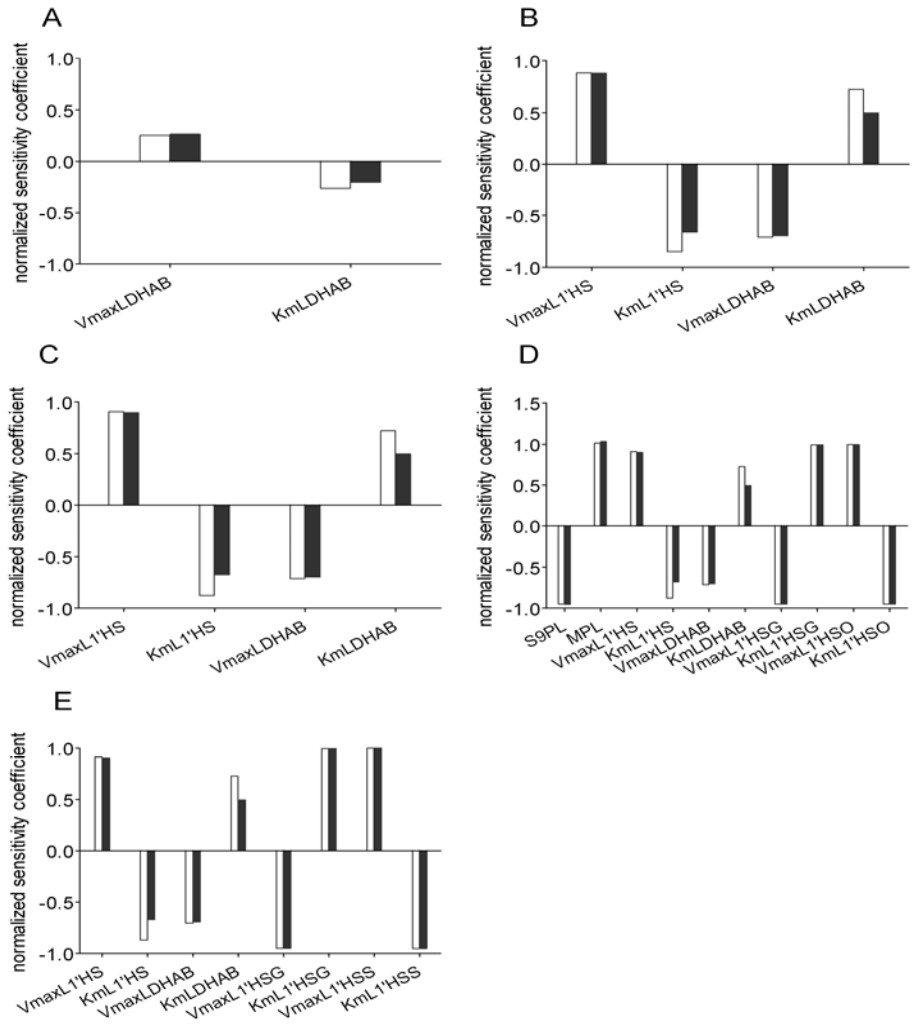

Figure 2.12. Sensitivity of the predicted formation of (A) 1,2-dihydroxy-4-allylbenzene, (B) 1′-hydroxysafrole, (C) 1′-hydroxysafrole glucuronide, (D) 1′-oxosafrole, and (E) 1′-sulfooxysafrole to different model parameters. White bars correspond to the normalized sensitivity coefficients at a dose of 0.05 mg/kg bw and black bars at a dose of 300 mg/kg bw. S9PL = Liver S9 protein yield, MPL = Liver microsomal protein yield, V_{max} and K_m are the maximum rate of formation and the Michaelis-Menten constant for the formation of the different metabolites in the liver: 1′-hydroxysafrole (1′HS), 1,2-dihydroxy-4-allylbenzene (DHAB), 1′-hydroxysafrole glucuronide (1′HSG), 1′-oxosafrole (1′HOS), and 1′-sulfooxysafrole (1′HSS).

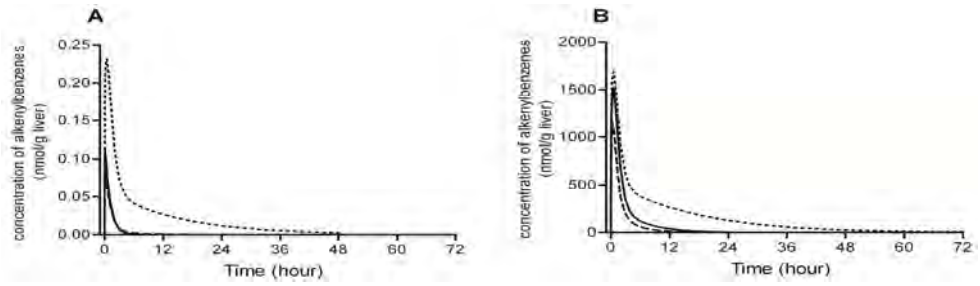

Figure 2.13. PBBK model predicted time dependent concentration (nmol/g liver) of estragole (——), methyleugenol (— —), and safrole (••••) at a dose level of (A) 0.05 mg/kg bw and (B) 300 mg/kg bw

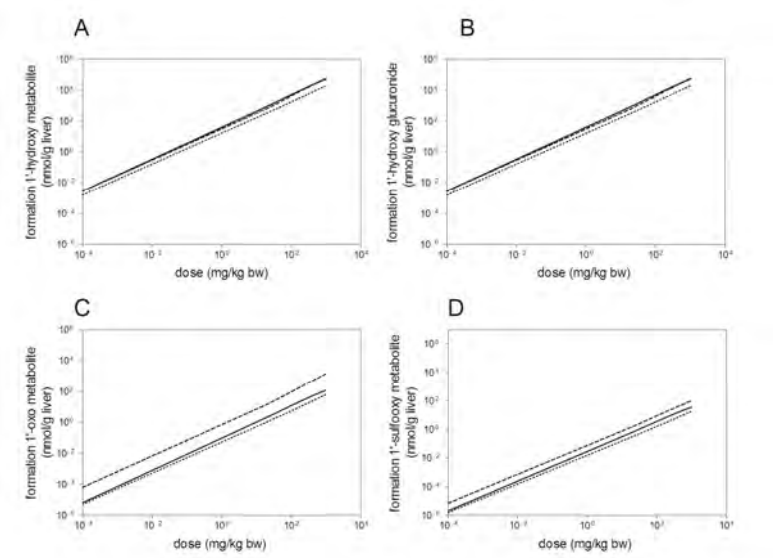

Figure 2.14. PBBK predicted dose dependent formation of (A) the 1′-hydroxy metabolite, (B) 1′-hydroxy glucuronide, (C) 1′-oxo metabolite, and (D) 1′-sulfooxy metabolite of estragole (———), methyleugenol (— — —), and safrole (••••) in male rat liver (Sprague-Dawley) after 24 h in case of estragole and methyleugenol and 72 h in case of safrole (Al-Subeihi *et al.*, 2011; Punt *et al.*, 2008).

Discussion

In the present study, a PBBK model was developed that allowed for the evaluation of bioactivation and detoxification of safrole at different oral dose levels in male rats. The advantages of PBBK models over in vivo or in vitro experiments are that PBBK models provide more insight into which tissues are involved, provide insight into the formation and distribution of metabolites in the various tissues of interest, allow predictions of the formation of the proximate and ultimate reactive unstable carcinogenic metabolite(s) and provide insight into the dose levels at which deviation from linear kinetics for bioactivation and detoxification might occur.

The performance of the newly developed PBBK model was evaluated by comparison of the predicted levels of metabolite formation to levels reported in the literature. A first experimental data set available reported levels for the formation of total safrole metabolites detected in the urine of Sprague-Dawley rats exposed to three different oral doses of safrole (Benedetti *et al.*, 1977). The model predicted the time dependent formation of safrole metabolites relatively well especially at dose levels of 0.63 and 60 mg/kg bw. A second experimental data set available for the evaluation of model performance reported time dependent excretion of 1,2-dihydroxy-4-allylbenzene and 1′-hydroxysafrole as 1′-hydroxysafrole glucuronide, in the urine of Wistar rats exposed to 162 mg/kg bw safrole (Klungsøyr and Scheline, 1983). The model predicted the formation of these two metabolites at levels amounting to, respectively, 74% and 10% of the dose, whereas the percentages experimentally observed amounted to respectively 72% and 4 % of the dose. Thus, the model appeared to accurately predict the biotransformation for safrole generally within 2-fold variation. Part of the deviations observed may be due to the fact that our PBBK model was developed using tissue samples from Sprague-Dawley rats, whereas the literature data were obtained with, respectively, Sprague-Dawley (Benedetti *et al.*, 1977) and Wistar rats (Klungsøyr and Scheline, 1983). Different species may have different types and contents of enzymes responsible for safrole metabolism, and metabolism may vary depending on the rat strain used (Kishida *et al.*, 2008). Moreover, uncertainty in the exact values of the model parameters for scaling up the in vitro data to the in vivo situation may affect the accuracy of the model as the sensitivity analysis revealed that the formation of

1,2-dihydroxy-4-allylbenzene and 1′-hydroxysafrole is mostly affected by kinetic parameters. Altogether, the results obtained in the analysis of model performance imply that conclusions derived from the model should preferably not be based on absolute outcomes but rather on a comparison of different conditions in a relative way as done in the present study.

Comparison of the PBBK model based predicted time dependent concentrations of the three alkenylbenzenes in the liver reveals that for safrole, in contrast to estragole and methyleugenol, metabolism might not be complete within 24 h upon dosing pointing at possibilities for accumulation upon repeated dosing especially at relatively high dose levels. The major detoxification route of safrole is O-demethylation resulting in 1,2-dihydroxy-4-allylbenzene (predicted to account for 74.5% of the dose at a dose of 0.05 mg/kg bw) a reaction catalyzed almost entirely in the liver. This result is different from the results obtained for the related alkenylbenzene estragole for which at a dose of 0.05 mg/kg bw, the major detoxification route, resulting in 4-allylphenol upon O-demethylation, appeared to occur predominantly in the lung and kidney (Punt *et al.*, 2008). Thus, at relatively low dose levels, extrahepatic metabolism has a role in rapid metabolism of estragole while this is not the case for safrole where metabolism mainly occurs in the liver, resulting in a longer time needed for complete conversion.

The PBBK predictions also revealed that the relative formation of the various safrole P450 mediated metabolites is hardly changing with the dose (Figure 2.11). The formation of the proximate carcinogenic metabolite 1′-hydroxysafrole is predicted to increase from 9.1% to 10.2% of the dose when the dose increases from 0.05 mg/kg bw to 300 mg/kg bw. Together, these data indicate that in contrast to outcomes previously obtained from similar PBBK models for the related alkenylbenzenes estragole and methyleugenol (Al-Subeihi *et al.*, 2011; Punt *et al.*, 2008) for safrole a dose dependent shift in P450 catalyzed reactions leading to a relative increase in bioactivation at higher dose levels is not observed.

The 1′-hydroxy metabolites of the alkenylbenzenes and their sulphate conjugates have been shown to form DNA adducts in vivo and in vitro in a dose dependent manner (Boberg *et al.*, 1983; Daimon *et al.*, 1998; Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Miller and

Miller, 1983; Phillips *et al.*, 1984; Randerath *et al.*, 1984; Swanson *et al.*, 1981). It is of interest to compare the results for the PBBK predicted formation of the ultimate carcinogenic metabolites of the three alkenylbenzenes to their relative potency for DNA adduct formation in vitro and in vivo and even to their $BMDL_{10}$ values (the lower confidence bound of the benchmark dose that gives 10% extra cancer incidence (BMD_{10})) for tumor formation. In HepG2 cells exposed to the alkenylbenzenes, the dose dependent formation of DNA adducts decreased in the order methyleugenol > estragole > safrole, but varied at most 3.5-fold (Zhou *et al.*, 2007). This is similar to the earlier finding in experiments in vivo in mouse liver in which the formation of DNA adducts in the liver of mice exposed to alkenylbenzenes also varied by less than 3.4-fold being again highest for methyleugenol (Randerath *et al.*, 1984). Furthermore, the estimated calculated $BMDL_{10}$ values of safrole, methyleugenol and estragole amount to approximately 3-29 mg/kg bw/day (calculated using BMD software based on the data from (Boberg *et al.*, 1983; Miller *et al.*, 1983)), 22-32 mg/kg bw (Rietjens *et al.*, 2008) and 9-33 mg/kg bw/day (Rietjens *et al.*, 2010), respectively. This indicates that these alkenylbenzenes have a comparable carcinogenic potential.

Altogether, it is concluded that the present study describes a PBBK model to quantify the bioactivation and detoxification of safrole in male rats. In contrast to outcomes previously obtained from similar PBBK models for the related alkenylbenzenes estragole and methyleugenol, for safrole a dose dependent shift in P450 mediated conversion leading to a relative increase in bioactivation at higher dose levels is not observed. In spite of this difference and the fact that complete metabolism of safrole takes longer than that of estragole and methyleugenol, the overall differences in bioactivation of the three alkenylbenzenes to their ultimate carcinogenic 1′-sulfooxy metabolites are limited, which is in line with the generally less than 4-fold difference in their level of DNA binding in in vitro and in vivo studies and in their comparable $BMDL_{10}$ values in in vivo carcinogenicity studies. It is concluded that in spite of differences in the rate of specific metabolic conversions, overall the levels of bioactivation of the three alkenylbenzenes is comparable, which is in line with their comparable carcinogenic potential.

Funding support

This research was funded by the Directorate General of Higher Education Ministry of National Education Republic of Indonesia.

Acknowledgments

The authors would like to thank Dr. Yiannis Fiamegos for synthesis of 1′-hydroxysafrole and Dr. Judy Bolton and the Department of Medicinal Chemistry and Pharmacognosy College of Pharmacy The University of Illinois at Chicago for providing synthesized 1,2-dihydroxy-4-allylbenzene.

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CHAPTER 3

Physiologically based biokinetic (PBBK) modeling of safrole bioactivation and detoxification in humans as compared with rats

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Based on: Toxicological Sciences (2012) 128, 301-316

Abstract

A physiologically based biokinetic (PBBK) model for the alkenylbenzene safrole in humans was developed based on in vitro- and in silico-derived kinetic parameters. With the model obtained, the time- and dose-dependent formation of the proximate and ultimate carcinogenic metabolites, 1′-hydroxysafrole and 1′-sulfooxysafrole in human liver were estimated and compared with previously predicted levels of these metabolites in rat liver. In addition, Monte Carlo simulations were performed to predict interindividual variation in the formation of these metabolites in the overall population. For the evaluation of the model performance, a comparison was made between the predicted total amount of urinary metabolites of safrole and the reported total levels of metabolites in the urine of humans exposed to safrole, which adequately matched. The model results revealed no dose-dependent shifts in safrole metabolism and no relative increase in bioactivation at dose levels up to 100 mg/kg body weight/day. Species differences were mainly observed in the detoxification pathways of 1′-hydroxysafrole, with the formation of 1′-oxosafrole being a main detoxification pathway of 1′-hydroxysafrole in humans but a minor pathway in rats, and glucuronidation of 1′-hydroxysafrole being less important in humans than in rats. The formation of 1′-sulfooxysafrole was predicted to vary 4- to 17-fold in the population (fold-difference between the 95th and median, and 95th and 5th percentile respectively), with the median being three to five times higher in human than in rat liver. Comparison of the PBBK results for safrole with those previously obtained for the related alkenylbenzenes estragole and methyleugenol revealed that differences in 1′-sulfooxy metabolite formation are limited, being only twofold to fivefold.

Introduction

Safrole, a natural plant constituent, is the major component of sassafras oil and a minor constituent in star anise, nutmeg and black pepper. Safrole was documented to be a rodent carcinogen (Borchert *et al.*, 1973; Miller *et al.*, 1983; Wislocki *et al.*, 1977). The genotoxicity and carcinogenicity of safrole has been related to the formation of DNA adducts (Borchert *et al.*, 1973; Daimon *et al.*, 1998). Studies on the bioactivation and detoxification of safrole in humans are limited. Chewing of betel quid, which contains *Piper betle* leaves containing 15 mg safrole/g fresh weight, has been reported to result in safrole concentrations of up to 420 µM in saliva (Hwang *et al.*, 1992). A case-control study showed a significant correlation between the incidence of oral squamous cell carcinoma and chewing of betel quid in a Taiwanese population (Ko *et al.*, 1995). This phenomenon was linked to a high frequency of safrole-DNA adducts in squamous cell carcinoma and oral submucous fibrosis tissues of betel quid users (Chen *et al.*, 1999).

Safrole and sassafras oil were banned as food additives in 1960 by the U.S. Food and Drug Administration (FDA). In Europe, a limitation of safrole in foodstuff and beverages was set at 1 ppm (The European Council Directive on food flavoring 88/388/EEC, amended by Commission Directive 91/71/EEC) (EEC, 1988). In 1997, the Committee of Experts on Flavoring Substances (CEFS) of the Council of Europe evaluated safrole and concluded that safrole is a weak hepatocarcinogen in experimental animal studies but also a genotoxic and a transplacental carcinogen; hence efforts should be made to reduce its consumption through foods and beverages as much as possible (Council of Europe, 1997). The Scientific Committee on Food (SCF) of the European Union concluded in their evaluation that safrole is genotoxic and carcinogenic and that reductions in the exposure and restriction in the use levels are indicated (SCF, 2002).

Daily intakes of safrole were estimated by the SCF to amount to 0.3 mg/day equivalent to 5 µg/kg body weight (bw)/day for a 60 kg person (SCF, 2002), and the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) estimated the maximum dietary exposure to safrole to amount to 879 µg/person/day equivalent to 14.6 µg/kg bw/day for a 60 kg person (JECFA, 2008). Furthermore, a study performed amongst non-betel quid chewers in

Taiwan showed that the urine of non-betel quid chewers contained 1,2-dihydroxy-4-allylbenzene, a major metabolite of safrole pointing out that intake of safrole from common spices in Taiwanese foods such as ginger and black pepper results in detectable exposure levels (Chang *et al.*, 2002).

The carcinogenicity of safrole has been correlated to its metabolic conversion by cytochromes P450 and sulfotransferases (SULTs) to a genotoxic metabolite, 1′-sulfooxysafrole (Borchert *et al.*, 1973; Ueng *et al.*, 2005; Wislocki *et al.*, 1977). In humans, the first step in this bioactivation is mainly catalyzed by cytochromes P450 2A6, P450 D6*1, P450 2C9, P450 2C19 and P450 2E1 (Jeurissen *et al.*, 2004; Liu *et al.*, 2004; Ueng *et al.*, 2004). The proximate carcinogenic metabolite 1′-hydroxysafrole can subsequently be sulfonated to the ultimate carcinogenic metabolite 1′-sulfooxysafrole, which is unstable and spontaneously decomposes to produce an electrophilic carbonium cation forming covalent adducts with cellular macromolecules including DNA (Borchert *et al.*, 1973; Ioannides *et al.*, 1981; Wislocki *et al.*, 1977). Other metabolites of safrole are presented in Figure 3.1 and include products resulting from *O*-demethylation, epoxidation and 3′-hydroxylation being 1,2-dihydroxy-4-allylbenzene, 2′,3′-safrole oxide and 3′-hydroxysafrole, respectively. In rats, 1,2-dihydroxy-4-allylbenzene is the major metabolite of microsomal conversion of safrole and this metabolite can subsequently be oxidized to an *o*-quinone leading to an electrophilic *p*-quinone methide intermediate (Bolton *et al.*, 1994). 2′,3′-Safrole oxide is susceptible to hydrolysis by microsomal epoxide hydrolase forming 2′,3′-dihydroxysafrole (Ioannides *et al.*, 1981; Klungsøyr and Scheline, 1983). 1′-Hydroxysafrole is converted to the ultimate carcinogen by SULT-mediated conversion to 1′-sulfooxysafrole, but can also be detoxified via oxidation to 1′-oxosafrole or glucuronidation to 1′-hydroxysafrole glucuronide (Figure 3.1) (Punt *et al.*, 2008).

Species-dependent differences in bioactivation and detoxification of safrole have been reported. For example, glucuronide conjugates of 1′-hydroxysafrole and 3′-hydroxysafrole were detected in the urine of rats but not in that of humans (Benedetti *et al.*, 1977). Formation of the 1′-oxo metabolites of the related alkenylbenzenes estragole and methyleugenol appears to be a minor detoxification pathway in rats (Al-Subeihi *et al.*, 2011; Martati *et al.*, 2011; Punt *et al.*, 2008). In humans, however, oxidation of the 1′-

hydroxy metabolite of the related alkenylbenzenes estragole and methyleugenol appeared to be a major pathway for detoxification of the 1′-hydroxy metabolite (Al-Subeihi *et al.*, 2012; Punt *et al.*, 2009).

To facilitate extrapolations of high dose animal carcinogenicity data for safrole to low dose in human exposure scenarios, insights are required in possible dose-dependent effects, species differences, and interindividual human variation in bioactivation and detoxification of this compound. The aim of the present study was to build a physiologically based biokinetic (PBBK) model for safrole in humans to obtain these insights. For the model development, the kinetic parameters for the metabolic conversions of safrole and 1′-hydroxysafrole were determined in vitro using tissue fractions of human organs that were found to be capable of metabolizing safrole or 1′-hydroxysafrole. The outcomes of the model were compared with those of the previously defined PBBK model for safrole in rats (Martati *et al*., 2011) to evaluate the occurrence of species differences in bioactivation and detoxification of safrole. In addition, Monte Carlo simulations were performed to predict interindividual variation in the formation of these metabolites in the population as a whole. Finally, the outcomes of the humans model were also compared with those obtained with previously defined PBBK models for the related alkenylbenzenes estragole and methyleugenol (Punt *et al.*, 2010; Al-Subeihi *et al.*, 2012).

Materials and methods

Materials. Pooled human liver microsomes, cytosol and S9 were purchased from BD Gentest (Worburn, MA). Pooled human lung and kidney microsomes were obtained from BioPredic International (Rennes, France). Safrole (purity 97%), 2′,3′-dihydroxysafrole (purity 99%), Tris(hydroxymethyl)aminomethane, alamethicin (from *Trichoderma viride*), L-ascorbic acid, uridine 5′-diphosphoglucuronic acid (UDPGA) and 3,4-(methylenedioxy) cinnamic acid were purchased from Sigma-Aldrich (Steinheim, Germany). β-Glucuronidase and reduced β-nicotinamide adenin dinucleotide phosphate (NADPH) and oxidized (NAD^+) were obtained from Roche Diagnostics (Mannheim, Germany). Reduced glutathione (GSH) and dimethyl sulfoxide (DMSO) were purchased from Acros Organics (New Jersey). 3′-Phosphoadenosine-5′-phosphosulphate (PAPS) was obtained from Sigma-

Aldrich (Zwijndrecht, The Netherlands). Potassium dihydrogen phosphate, dipotassium hydrogen phosphate trihydrate, acetic acid (glacial) and magnesium chloride were purchased from VWR (Darmstadt, Germany). Chromatography grade acetonitrile was purchased from Biosolve (Valkenswaard, The Netherlands). 1′-Hydroxysafrole was synthesized and purified as described previously (Jeurissen *et al.*, 2004; Martati *et al.*, 2011). 3′-Hydroxysafrole was synthesized from 3,4-(methylenedioxy) cinnamic acid as described previously (Angle *et al.*, 2008) and purified as previously described (Martati *et al.*, 2011). 1′-Oxosafrole was synthesized from 1′-hydroxysafrole based on the method used for 1′-oxoestragole (Wislocki *et al.*, 1976). 1,2-Dihydroxy-4-allylbenzene was synthesized as described previously (Bolton *et al.*, 1994).

Determination of the kinetic constants for microsomal conversion of safrole

Metabolites formed by microsomal conversion of safrole were identified and quantified upon incubation of safrole with human liver, lung, and kidney microsomes in the presence of NADPH. These incubations were performed as described previously for the microsomal conversion of safrole in rat tissue fractions (Martati *et al.*, 2011). The incubation mixtures had a final volume of 160 µL and contained 1 mg/mL microsomal protein, 3 mM NADPH, and 1 mM ascorbic acid in 0.2 M Tris-HCl (pH 7.4). When metabolite formation was observed with a specific tissue fraction, the kinetic constants for the formation of this metabolite were determined by performing incubations for 10 min at 37°C at substrate concentrations that ranged from 10 to 1000 µM (final concentration). The reactions were started after 1 min of pre-incubation by addition of the substrate and terminated by addition of 40 µL of cold acetonitrile. An incubation mixture without NADPH was used as the blank.

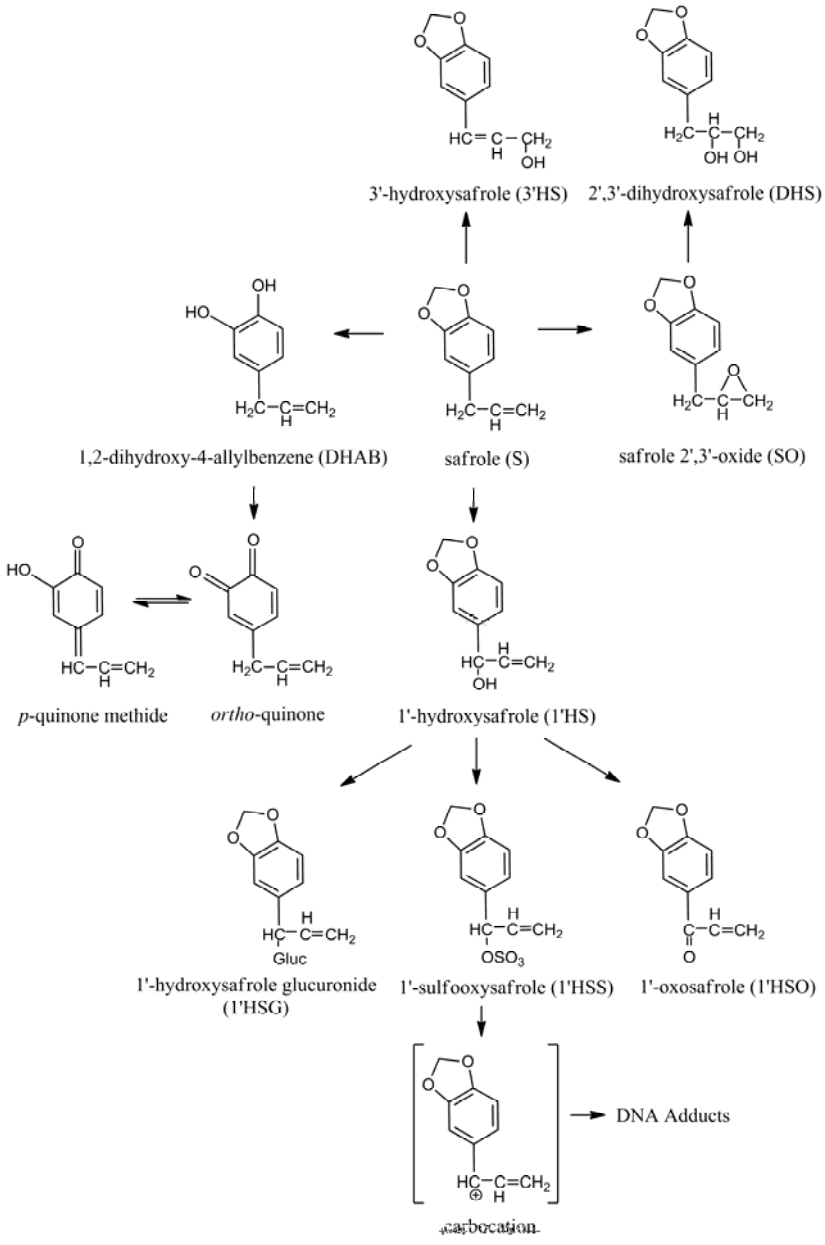

Figure 3.1. Biotransformation pathways of safrole.

Determination of kinetic constants for glucuronidation, sulfation, and oxidation of 1′-hydroxysafrole

To determine the kinetic constants for the metabolic conversion of 1′-hydroxysafrole to 1′-hydroxysafrole glucuronide, 1′-sulfooxysafrole, and 1′-oxosafrole, incubations were performed with human liver S9 (in case of glucuronidation and sulfonation) or human liver microsomes (in case of oxidation) in the presence of the relevant cofactors. The choice of the tissue fractions was based on our previous works on the related alkenylbenzene estragole (Punt *et al*., 2008; Punt *et al*., 2009) in order to make the results comparable. Incubations for glucuronidation and sulfonation were, for instance, performed with S9 fractions and not with microsomes and cytosol separately to allow for direct comparison of the kinetic constants obtained for these reactions (Punt *et al*., 2007; Punt *et al*., 2008). All the reactions were performed as described previously for the metabolic conversion of 1′-hydroxysafrole by rat tissue fractions (Martati *et al.*, 2011). In case of glucuronidation, the incubation mixtures had a final volume of 200 µL and contained (final concentrations) 10 mM UDPGA, 0.2 mg S9 protein/mL in 0.2 M Tris-HCl (pH 7.4) and 10 mM $MgCl_2$. These incubation mixtures were first pre-treated on ice with 0.025 mg/mL alamethicin for 15 min to obtain maximum glucuronidation activity (Fisher *et al.*, 2000). The reactions were carried out for 360 min at 37°C at a substrate concentration that ranged from 100 to 4000 µM (final concentration). In case of sulfonation of 1′-hydroxysafrole the incubation mixtures had a final volume of 100 µL, containing (final concentrations) 0.2 mM PAPS, 2 mg S9 protein/mL, and 10 mM GSH in 0.1 M potassium phosphate (pH 8.0). These incubations were carried out for 120 min at 37°C at a substrate concentration that ranged from 250 to 4000 µM (final concentration). For oxidation of 1′-hydroxysafrole, initial incubations were performed to determine the subcellular distribution and enzyme specificity of this enzymatic reaction. These reactions were carried out for 10 min at a substrate concentration of 1000 µM with incubation mixtures that had a final volume of 100 µL, containing (final concentrations) 3 mM NAD^+, NADH, $NADP^+$, or NADPH, 1 mg microsomal or cytosolic protein/mL, and 2 mM GSH in 0.2 M Tris-HCl (pH 7.4). Based on these incubations, the oxidation reaction of 1′-hydroxysafrole was observed to mainly occur in incubations with microsomes and NAD^+ as cofactor (data not shown) and the kinetic

constants were determined for this reaction only. For the determination of these kinetic constants, incubations were carried out for 10 min at 37°C at the same conditions as described above and a substrate concentration that ranged from 10 to 4000 µM (final concentration).

All of the above reactions were started after pre-incubation at 37°C for 1 min by addition of the substrate 1′-hydroxysafrole diluted in DMSO and terminated by addition of cold acetonitrile amounting to 25% (v/v) of the incubation volume. The final concentration of DMSO in the incubation mixtures was kept constant at 1%. Incubation mixtures without the specific cofactors were used as blanks.

Samples and data analysis

For the determination of kinetic constants for each metabolite, three replicates were always performed. All samples were centrifuged for 5 min at 16000 g and the supernatants were frozen at -20°C until analysis by high performance liquid chromatography (HPLC) or ultra performance liquid chromatography (UPLC). Analysis of the samples resulting from the microsomal conversion of safrole as well as the oxidation and glucuronidation of 1′-hydroxysafrole was performed on a Waters M600 HPLC equipped with a photodiode array detector as previously described (Martati *et al.*, 2011). Quantification and detection of the GSH conjugate of 1′-sulfooxysafrole were performed on a Waters UPLC equipped with a photodiode array detector system as described previously (Martati *et al.*, 2011). The different metabolites of safrole and 1′-hydroxysafrole were identified and quantified on the basis of the similarity of their UV spectrum and retention time and compared with the UV spectrum and retention time of synthesized or commercially available reference compounds as described previously (Martati *et al.*, 2011). In the microsomal incubations, safrole 2′,3′-oxide was not detected because of its swift hydrolysis by microsomal epoxide hydrolase to 2′,3′-dihydroxysafrole (Guenthner and Luo, 2001; Luo *et al.*, 1992; Stillwell *et al.*, 1974).

The safrole concentration-dependent rate of formation of different products of microsomal conversion of safrole, and the 1′-hydroxysafrole concentration-dependent rate of glucuronidation, oxidation and sulfation were fitted to the standard Michaelis-Menten equation: $v = V_{max}*[S]/(K_m+[S])$. In this equation, [S] represents the substrate concentration

of the respective reaction. The apparent maximum velocity ($V_{max(app)}$) and apparent Michaelis-Menten constant ($K_{m(app)}$) were determined using GraphPad Prism version 5.03 (GraphPad Software, San Diego CA). The values of V_{max} and K_m were reported in nmol/min/mg microsomal or S9 protein and µM, respectively. Scaling of in vitro data, expressed as nmol/min/mg protein, to in vivo data, expressed as µmol/h/g tissue, was performed as described below.

Definition of the PBBK model for safrole metabolism

A PBBK model describing the dose-dependent kinetic behavior of safrole and formation of its metabolites in humans was developed based on the model previously defined for safrole in male rats (Martati *et al.*, 2011), and for the related alkenylbenzene estragole in rats (Punt *et al*., 2008) and in humans (Punt *et al*., 2009). Figure 3.2 presents a schematic overview of the PBBK model used to simulate the metabolism and distribution of safrole in humans. The following compartments were included in the model: (1) liver and kidney as metabolizing compartments, and (2) lung, fat, blood, richly perfused tissue and slowly perfused tissue as separate compartments. No parallel model describing the distribution of 1′-hydroxysafrole over the body was included, because a near quantitative intrahepatic conversion of 1′-hydroxysafrole was assumed. This assumption was based on the findings (see Results section) that the overall catalytic efficiency for the reactions converting 1′-hydroxysafrole was higher than the catalytic efficiency for the formation of 1′-hydroxysafrole.

The uptake of safrole from the gastrointestinal tract was assumed to follow first-order kinetics, assuming safrole to be absorbed directly to the liver with an estimated absorption constant set at 1.0 h^{-1}. This assumption was based on the fact that uptake of the related alkenylbenzene estragole from the gastrointestinal track is known to be rapid and complete with an estimated absorption half-life of 0.7 h (Anthony *et al.*, 1987; Punt *et al.*, 2008). 3′-Hydroxysafrole formation was modeled in the liver and kidney. Microsomal metabolism of safrole resulting in 1′-hydroxysafrole, 2′,3′-dihydroxysafrole (derived from safrole 2′,3′-oxide) and 1,2-dihydroxy-4-allylbenzene was only observed in the liver and therefore modeled only in the liver compartment. Formation of 1′-hydroxysafrole in the liver was

followed by its further conversion to 1′-hydroxysafrole glucuronide, 1′-sulfooxysafrole, and 1′-oxosafrole. The mass balance equations in the PBBK model for time-dependent changes of safrole in the liver and kidney were essentially the same as previously described for the rats PBBK model (Martati *et al.*, 2011).

The urinary excretion of safrole metabolites was considered rapid and complete based on the literature data of Benedetti *et al.* (1977) indicating that ^{14}C-safrole administered to humans was excreted almost completely in the urine within 5 days, and that only a limited percentage of the dose (< 2.25%) was found in feces. Based on this consideration, the transport of metabolites from liver to the kidney and urine was not included in the model. Instead, the metabolites 2′,3′-dihydroxysafrole, 1,2-dihydroxy-4-allylbenzene, 1′-hydroxysafrole glucuronide and 1′-oxosafrole formed in liver and 3′-hydroxysafrole formed in the liver and kidney were assumed to be all completely excreted in the urine.

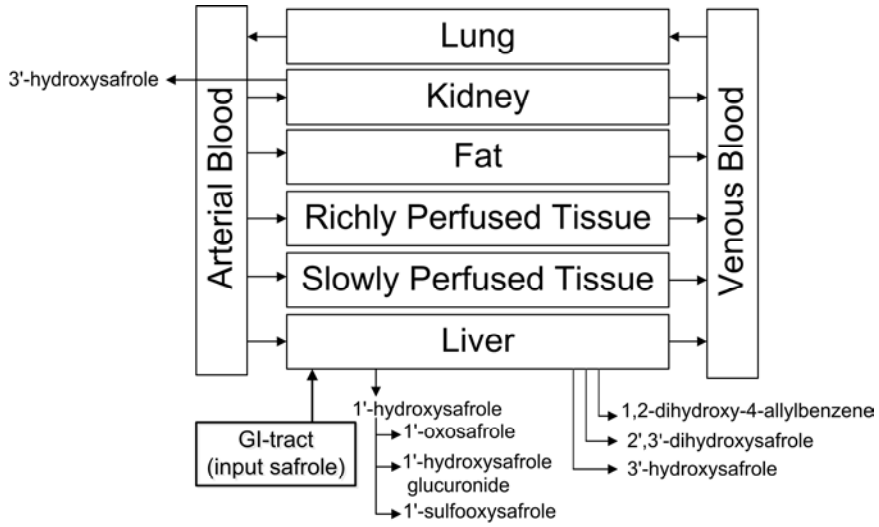

Figure 3.2. Schematic presentation of the physiologically based biokinetic (PBBK) model used to simulate the bioactivation and detoxification of safrole in humans.

The kinetic parameters for the product formation upon by microsomal conversion of safrole and for the glucuronidation, oxidation and sulfation of 1′-hydroxysafrole were determined in vitro. The V_{max} values for the formation of microsomal safrole metabolites and of conjugated metabolites obtained from incubations with microsomal liver samples were scaled to the liver using a microsomal protein yield of 32 mg/g liver (Barter *et al*., 2007). The V_{max} values for the formation of metabolites formed in the kidney were scaled using a microsomal protein yield of 7 mg/g kidney (Beierschmitt and Weiner,1986; Medinsky *et al.*, 1994; Punt *et al.*, 2008). The V_{max} values for the sulfation and glucuronidation of 1′-hydroxysafrole were scaled to the liver using an S9 protein yield of 143 mg/g liver (Medinsky *et al.*, 1994; Punt *et al.*, 2008; Al-Subeihi *et al*., 2012).

The physiological parameter values including organ volumes, cardiac output, and blood flows were taken from literature (Brown *et al.*, 1997) and are shown in Table 3.1.

Table 3.1. Parameters used in the physiologically based biokinetic model for safrole in humans.

Physiological Parameters[a]		Tissue : Blood Partition Coefficients[b]	
Body Weight (kg)	60		
Percentage of Body Weight		Safrole	
Liver	2.6	Liver	6.7
Kidney	0.4	Kidney	6.7
Fat	21.4	Fat	106
Rapidly Perfused	4.6	Rapidly Perfused	6.7
Slowly Perfused	51.7	Slowly Perfused	4.2
Blood	7.9	1′-Hydroxysafrole	
		Liver	1.7
Cardiac Output (L/h/kg $bw^{0.74}$)	15.0		
Percentage of Cardiac Output			
Liver	22.7		
Kidney	17.5		
Fat	5.2		
Rapidly Perfused	29.8		
Slowly Perfused	24.8		

[a] Brown *et al.* (1997)

[b] DeJongh *et al.* (1997)

Partition coefficients for safrole and 1′-hydroxysafrole were estimated from the ClogP values (DeJongh *et al.*, 1997). ClogP values were calculated with ChemBio3D Ultra 2010 (CambridgeSoft, MA) and were 3.18 and 1.62 for safrole and 1′-hydroxysafrole, respectively. Mass balance and differential equations of safrole metabolism were coded and simulated with Berkeley Madonna 8.3.14 (Macey and Oster, UC Berkeley, CA) using the Rosenbrock′s algorithm for stiff system. PBBK model predictions of the dose-dependent formation of the various products of microsomal conversion of safrole and of the various conversion products of 1′-hydroxysafrole were performed over 120 h. In our PBBK model predictions, 120 h was selected because for this time point in vivo human experimental data that could be used for model evaluation were available (Benedetti *et al.*, 1977).

Sensitivity analysis

Sensitivity analysis was used to identify the parameters that have the greatest impact on a specified model output (DeWoskin, 2007). Normalized sensitivity coefficients (SC) were determined according to the following equation: SC = (C′- C)/(P′-P)*(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′ is the modified parameter (Rietjens *et al.*, 2011). The effect of a 5% change in each parameter on the formation of 1′-hydroxysafrole and 1′-sulfooxysafrole (expressed as percentage of dose over 120 h) was analyzed. The final coefficients were calculated using Excel (Excel 2007, Microsoft Corporation, WA).

Evaluation of interindividual variability on population level using Monte Carlo simulation

To predict the influence of interindividual variability on the model outcomes, a Monte Carlo simulation was performed. For this simulation, a total number of 10,000 simulations were run. The parameters that significantly affect the outcomes as indicated by the normalized SC values higher than |0.1| were included in the Monte Carlo simulations. For these simulations, the mean μ_w and standard deviation σ_w describing the log-normal

distribution of each parameter were derived from the mean μ_x and coefficient of variation of the non-ln-transformed data using the following equation (Zhang *et al.*, 2007) :

$$\mu_w = \ln\left[\mu_x \Big/ \sqrt{\left(1+CV_x{}^2\right)}\right] \qquad \text{and} \qquad \sigma_w{}^2 = \ln\left(1+CV_x{}^2\right)$$

Meanwhile, the values of the parameters that have normalized SC values lower than |0.1| were kept at their mean values. The coefficients of variation (CVs) of the distribution of the yield of S9 protein (S9PL), microsomal protein yield of the liver (MPL), and volume of the liver (VL) were collected from the literature and corresponded to 46% (Barter *et al.*, 2007), 46% (Barter *et al.*, 2007), and 5% (EPA, 2000), respectively. For the kinetic constants of the different metabolic reactions included in the Monte Carlo simulations, general CVs of 30% were used to represent a moderate level of uncertainty and variability (Covington *et al.*, 2007). The outputs were then analyzed statistically to calculate the different percentiles of the distribution of the model outcomes using GraphPad Prism version 5.03 (GraphPad Software, San Diego CA).

Comparison of PBBK model-based predictions for safrole with the PBBK model-based predictions for the related alkenylbenzenes estragole and methyleugenol

The PBBK model-based predictions obtained for safrole were compared with those obtained with previously developed PBBK models for the related alkenylbenzenes estragole and methyleugenol (Al-Subeihi *et al.*, 2012; Punt *et al.*, 2009). To this end, the three PBBK models were run to obtain the dose-dependent formation of the respective 1′-hydroxy metabolites, 1′-hydroxy glucuronide metabolites, 1′-oxo metabolites, and 1′-sulfooxy metabolites. The PBBK models were run from dose levels of 10^{-6} mg/kg bw to 100 mg/kg bw for 120 h. The lowest dose level of 10^{-6} mg/kg bw was chosen to ascertain that the modeling would include realistic human dietary intake levels and the high dose levels of 100 mg/kg bw was chosen to make sure the evaluation would include dose levels known to cause tumors in in vivo rodent studies.

Results

Microsomal conversion of safrole

HPLC analysis of incubations with pooled human liver, kidney and lung microsomes in the presence of safrole as substrate and NADPH as cofactor reveals that conversion of safrole only occurs in incubations with pooled human liver and kidney microsomes, whereas no conversion of safrole occurs in incubations with human lung microsomes (data not shown). The metabolites formed in incubations with human liver microsomes are the same as those previously shown to be formed in incubations with rat liver microsomes (Martati *et al.*, 2011). Incubations of safrole with human kidney microsomes revealed the formation of only 3′-hydroxysafrole (chromatogram not shown). Altogether the data indicate that extrahepatic microsomal conversion of safrole contributes to a minor extent to the overall microsomal conversion of safrole.

Kinetics of microsomal conversion of safrole

Figure 3.3A shows the rate of formation of 2′,3′-dihydroxysafrole, 1′-hydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene in incubations with pooled human liver microsomes and increasing concentrations of safrole from 10 to 1000 µM. Similar experiments were performed with kidney microsomes using substrate concentrations ranging from 250 to 4000 µM (Figure 3.3B). Table 3.2 presents the apparent K_m and V_{max} values obtained from these plots and the catalytic efficiencies (V_{max}/K_m) derived from them.

The results obtained reveal that with pooled human liver microsomes 1,2-dihydroxy-4-allylbenzene is the major metabolite formed, followed by 1′-hydroxysafrole, 2′,3′-dihydroxysafrole and 3′-hydroxysafrole. The catalytic efficiencies of the major metabolites 1,2-dihydroxy-4-allylbenzene and 1′-hydroxysafrole, were 4.9 and 4.3 µL/min/mg microsomal protein, respectively.

The results obtained with human kidney microsomes reveal that only 3′-hydroxysafrole is formed. The apparent V_{max}, K_m and catalytic efficiency calculated as the apparent V_{max}/K_m for the formation of 3′-hydroxysafrole were 0.03 nmol/min/mg microsomal protein, 1054 µM, and 0.03 µL/min/mg microsomal protein,respectively (Figure 3.3B). In the kidney, 3′-

hydroxysafrole was also formed in the blank incubations without NADPH. Correcting the amount of 3′-hydroxysafrole formed in the complete incubation for the amount of 3′-hydroxysafrole formed in the blank incubation contributed to the relatively large experimental variation in the K_m value revealing a coefficient variation of 80%.

Glucuronidation of 1′-hydroxysafrole

Formation of the glucuronosyl conjugate of 1′-hydroxysafrole was analyzed in incubations with pooled human liver S9 and UDPGA as cofactor. Formation of the glucuronosyl conjugate of 1′-hydroxysafrole eluting at a retention time of 24 min was observed (chromatogram not shown). This metabolite was previously identified as 1′-hydroxysafrole glucuronide by liquid chromatography-mass spectrometry (LC-MS) (Martati *et al.*, 2011). Treatment of the sample with β-glucuronidase resulted in complete elimination of this metabolite and a concomitant increase in the intensity of the peak of 1′-hydroxysafrole, corroborating that the metabolite formed corresponds to the glucuronosyl conjugate of 1′-hydroxysafrole.

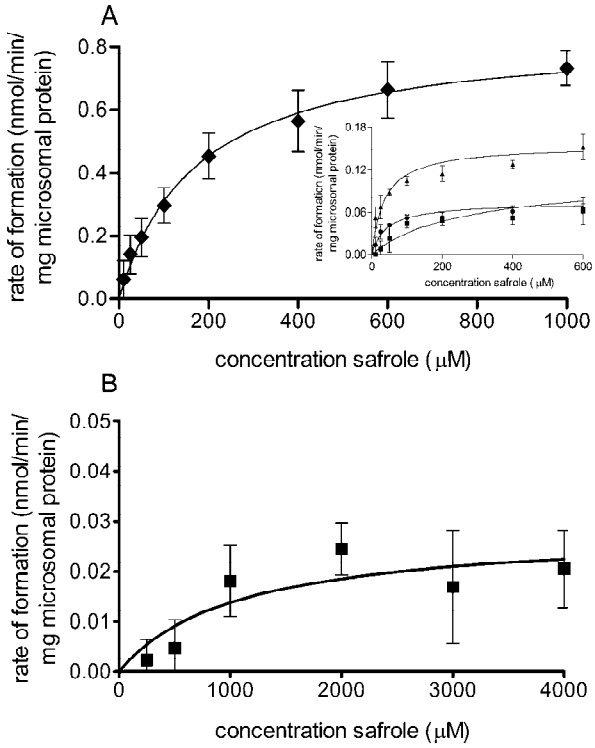

Figure 3.3. Safrole concentration dependent rate of formation of microsomal conversion of safrole in incubations with human (A) liver and (B) kidney microsomes and NADPH as a cofactor. In the plots each point represents the mean (± SD) of three replicates corresponding to the formation of 2′,3′-dihydroxysafrole (•), 1′-hydroxysafrole (▲), 3′-hydroxysafrole (■) and 1,2-dihydroxy-4-allylbenzene (◆).

Table 3.2. The kinetic parameters for microsomal conversion of safrole in incubations with liver and kidney microsomes and for conversion of 1′-hydroxysafrole in incubations with liver microsomes or S9 protein from humans and the cofactor for the respective conversions.

Organ	Metabolite	K_m (μM)[a]	Coefficient Variation of K_m	V_{max} (nmol/min/mg microsomal protein or S9 protein)[a]	Coefficient Variation of V_{max}	In vitro catalytic efficiency ($V_{max(app)}/K_m$) (μL/min/mg microsomal protein or S9 protein)
Microsomal conversion of safrole						
Liver	2′,3′-dihydroxysafrole	41 ± 10	24%	0.07 ± 0.004	6%	1.7
	1′-hydroxysafrole	35 ± 10	29%	0.15 ± 0.008	5%	4.3
	3′-hydroxysafrole	255 ± 99	39%	0.11 ± 0.01	9%	0.4
	1,2-dihydroxy-4-allylbenzene	172 ± 30	17%	0.85 ± 0.05	6%	4.9
Kidney	3′-hydroxysafrole	1054 ± 843	80%	0.03 ± 0.008	27%	0.03
Conversion of 1′-hydroxysafrole						
Liver	1′-hydroxysafrole glucuronide[b]	1322 ± 208	16%	0.1 ± 0.006	6%	0.08
	1′-oxosafrole [c]	549 ± 84	15%	7.5 ± 0.4	5%	13.7
	1′-sulfooxysafrole [b]	3828 ± 1801	47%	0.017 ± 0.005	29%	0.004

[a] The values are mean ± SD of three independent determinations; [b] Incubation with S9 protein; [c] Incubation with microsomal protein

Figure 3.4A shows the rate of glucuronidation of 1′-hydroxysafrole with a substrate concentration increasing from 100 to 4000 µM. The apparent V_{max} and K_m values derived from these data amounted to 0.1 nmol/min/mg S9 protein and 1322 µM, respectively, resulting in an apparent catalytic efficiency (V_{max}/K_m) of 0.08 µL/min/mg S9 protein (Table 3.2).

Oxidation of 1′-hydroxysafrole

Formation of the glutathione adduct with 1′-oxosafrole (GS-1′-oxosafrole) was analyzed in incubations with pooled human liver microsomes and NAD^+ as cofactor and GSH as a trapping agent for the 1′-oxosafrole formed. Formation of a single metabolite with a retention time of 12 min was observed (chromatogram not shown). This metabolite was previously identified by LC-MS as GS-1′-oxosafrole (Martati *et al.*, 2011).

Figure 3.4B shows the rate of oxidation of 1′-hydroxysafrole with increasing substrate concentrations ranging from 10 to 4000 µM. The apparent V_{max} obtained from this graph amounted to 7.5 nmol/min/mg microsomal protein and the apparent K_m value to 549 µM, resulting in an apparent catalytic efficiency (V_{max}/K_m) of 13.7 µL/min/mg microsomal protein (Tabel 3.2).

Sulfation of 1′-hydroxysafrole

Formation of the glutathione adduct with 1′-sulfooxysafrole (GS-1′-sulfooxysafrole) was analyzed in incubations with human liver S9 using PAPS as cofactor and GSH as trapping agent for the reactive carbocation derived from the unstable 1′-sulfooxysafrole metabolite. A single metabolite was formed which eluted at a retention time of 1.2 min (chromatogram not shown). This metabolite was previously identified as GS-1′-sulfooxysafrole by LC-MS (Martati *et al.*, 2011). UPLC analysis of incubations of 1′-sulfooxysafrole in the absence of GSH and in the presence of S9 protein and PAPS revealed no peak formation at 1.2 min (chromatograms not shown).

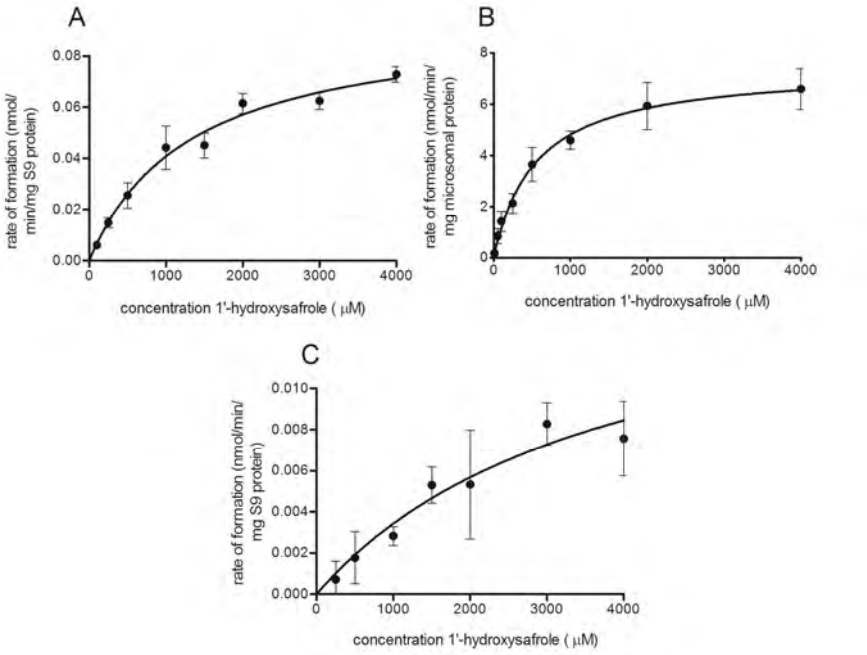

Figure 3.4. 1′-Hydroxysafrole concentration dependent rate of formation of (A) 1′-hydroxysafrole glucuronide with pooled human liver S9 in the presence of UDPGA as a cofactor, (B) GS-1′-oxosafrole with pooled human liver microsomes in the presence of NAD^+ as a cofactor and GSH as a trapping agent and (C) GS-1′-sulfooxysafrole with pooled human liver S9 homogenates in the presence of PAPS as a cofactor and GSH as a trapping agent. In the plot each point represents the mean (± SD) of three replicates

Figure 3.4C shows the rate of the formation of GS-1′-sulfooxysafrole with a substrate concentration increasing from 250 to 4000 µM. Based on these data the K_m for formation of 1′-sulfooxysafrole by human liver S9 protein was determined to be 3828 µM and the V_{max} 0.017 nmol/min/mg S9 protein resulting in a catalytic efficiency (V_{max}/K_m) of 0.004 µL/min/mg S9 protein (Table 3.2).

Table 3.3. Scaled kinetic parameters for safrole metabolism by human and male rat tissue fractions as used in the PBBK models.

Metabolite	Organ	Humans[a,b]			Male Rats[a,c]		
		Scaled $V_{max,\ in\ vivo}$ (μmol/h/g tissue)[d]	K_m (μM)	Catalytic efficiency (mL/h/g tissue)[e]	Scaled $V_{max,\ in\ vivo}$ (μmol/h/g tissue)[d]	K_m (μM)	Catalytic efficiency (mL/h/g tissue)[e]
Microsomal conversion of safrole							
2′,3′-dihydroxysafrole	Liver	0.1	41	2.4	0.20	149	1.3
1′-hydroxysafrole	Liver	0.3	35	8.6	0.8	497	1.6
	Lung	n.d	n.d	-	0.05	159	0.3
3′-hydroxysafrole	Liver	0.2	255	0.8	0.7	546	1.1
	Lung	n.d	n.d	-	0.1	3573	0.03
	Kidney	0.01	1054	0.01	0.04	3067	0.01
1,2-dihydroxy-4-allylbenzene	Liver	1.6	172	9.3	4.0	313	13.0
Conversion of 1′-hydroxysafrole							
1′-hydroxysafrole glucuronide	Liver	0.90	1322	0.7	215	101	2124
1′-oxosafrole	Liver	16.0	549	29	11.0	1809	6.3
1′-sulfooxysafrole	Liver	0.15	3828	0.04	0.26	127	2.0

[a] The values are mean ± SD of three independent determinations
[b] data obtained from present study
[c] data obtained from previous study
[d] Scaled V_{max} were converted from in vitro V_{max} based on microsomal protein yield of 32, 20 and 7 mg/g tissue for liver, lung and kidney, respectively, and S9 protein yield of 143 mg/g liver
[e] Catalytic efficiency (scaled V_{max}/K_m)
n.d : not detected

Comparison of kinetic data for safrole bioactivation and detoxification by humans and male rats

Table 3.3 presents a summary of the kinetic parameters for safrole bioactivation and detoxification by human tissue fractions compared to the kinetic parameters previously reported for male rat tissue fractions (Martati *et al.*, 2011). The V_{max} values expressed as µmol/h/g tissue were obtained by scaling the in vitro V_{max} values to V_{max} values expressed per g liver using microsomal or S9 protein yields from the different organs that were obtained from literature (Medinsky *et al.*, 1994). This conversion allowed comparison of the kinetic constants obtained with different tissue fractions.

In the human liver, the scaled V_{max} for formation of 1′-hydroxysafrole is about 2.5-fold lower than that for rat liver, whereas the apparent K_m is 14-fold lower than that for rat liver. Overall, the catalytic efficiency (scaled V_{max}/K_m) for formation of the proximate carcinogenic metabolite 1′-hydroxysafrole in human liver is 5 times higher than that in rat liver. The catalytic efficiency for formation 1,2-dihydroxy-4-allylbenzene is 1.3-fold lower in human than in male rat liver. The catalytic efficiency for formation of 2′,3′-dihydroxysafrole in human liver is 2.0-fold higher than that in rat liver. The catalytic efficiencies for formation of 3′-hydroxysafrole are similar for human and male rat liver.

Comparison of the catalytic efficiency for glucuronidation of 1′-hydroxysafrole by human and male rat liver reveals that this conversion is more than 3200 times less efficient in human liver which is due to a 250-fold lower scaled V_{max} and a 13-fold higher K_m for glucuronidation in human liver. Comparison of oxidation of 1′-hydroxysafrole in human liver to that in male rat liver reveals that this metabolic pathway is four times more efficient in human liver due to a 1.3-fold higher scaled V_{max} value and a 3.3-fold lower K_m value for 1′-hydroxysafrole oxidation in human liver as compared to rat liver. The catalytic efficiency for formation of 1′-sulfooxysafrole was 50-fold lower in human liver than in rat liver due to a 2-fold lower V_{max} value and a 30-fold higher K_m value.

Sensitivity analysis of the PBBK model developed

To identify the key parameters that affect the levels of the proximate carcinogenic metabolite 1′-hydroxysafrole and the ultimate carcinogenic metabolite 1′-sulfooxysafrole, a sensitivity analysis was performed. Normalized SC values (sensitivity coefficients) were calculated at a dose 0.005 mg/kg bw and only parameters that had a normalized SC higher than 0.1 (in absolute value) are presented (Figure 3.5). Formation of 1′-hydroxysafrole is predicted to be mostly affected by the kinetic constants for its formation from safrole ($V_{max,L\_1'HS}$ and $K_{m,L\_1'HS}$), the kinetic constants for formation for 2′,3′-dihydroxysafrole (V_{max,L_DHS} and K_{m,L_DHS}), the kinetic constants for formation of 1,2-dihydroxy-4-allylbenzene (V_{max,L_DHAB} and K_{m,L_DHAB}), microsomal protein yield of the liver (MPL), and the volume of the liver (VL). The predicted formation of 1′-sulfooxysafrole was mostly affected by the kinetic constants for formation of 1′-hydroxysafrole ($V_{max,L\_1'HS}$ and $K_{m,L\_1'HS}$), for oxidation of 1′-hydroxysafrole ($V_{max,L\_1'HOS}$ and $K_{m,L\_1'HOS}$) and for sulfation of 1′-hydroxysafrole ($V_{max,L\_1'HSS}$ and $K_{m,L\_1'HSS}$) and to a minor extent by the kinetic constants for formation of 1,2-dihydroxy-4-allylbenzene (V_{max,L_DHAB} and K_{m,L_DHAB}), for formation of 2′,3′-dihydroxysafrole (V_{max,L_DHS} and K_{m,L_DHS}), the yield of S9 protein (S9PL), the yield of microsomal protein of the liver (MPL), and the volume of the liver (VL).

Based on the sensitivity analysis probability distributions were assigned to the model parameters to which the model outcome is most sensitive, thereby allowing to perform Monte Carlo simulations to see the effect of uncertainty and variability on the PBBK based predictions for the total amount of metabolites excreted in the urine, for the formation of microsomal metabolites of safrole (2′,3′-dihydroxysafrole, 1′-hydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene), for the conversion of 1′-hydroxysafrole (1′-hydroxysafrole glucuronide, 1′-oxosafrole and 1′-sulfooxysafrole), for concentration of safrole in liver and blood and for the concentration of 1′-hydroxysafrole in the liver, and the predicted formation of 1′-sulfooxysafrole at different oral doses.

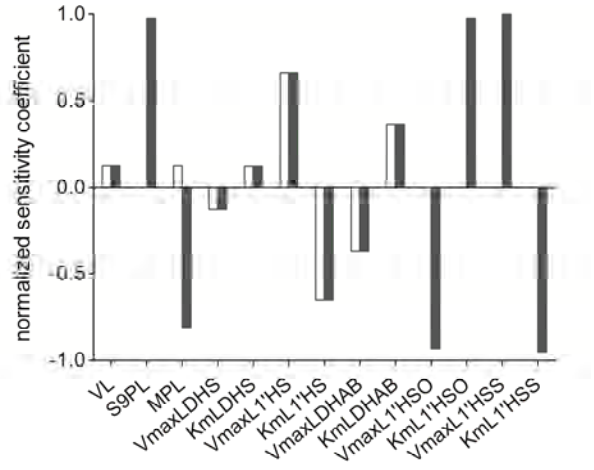

Figure 3.5. Sensitivity analysis showing the sensitivity of the predicted formation of 1′-hydroxysafrole (white bars) and 1′-sulfooxysafrole (black bars) to different model parameters. VL= volume of the liver, S9PL= liver S9 protein yield, MPL= microsomal protein yield of the liver and V_{max} and K_m are the maximum rate and the Michaelis-Menten constant for the formation of the different metabolites in the liver, respectively, 2′,3′-dihydroxysafrole (DHS), 1′-hydroxysafrole (1′HS), 1,2-dihydroxy-4-allylbenzene (DHAB), 1′-oxosafrole (1′HOS), and 1′-sulfooxysafrole (1′HSS).

Model simulation of total metabolites formed

Performance of the PBBK model thus obtained was evaluated by comparison of the PBBK-model based prediction for the total formation of metabolites that will be excreted in the urine to the reported total urinary excretion of safrole metabolites expressed as a percentage of the dose reported in the literature for humans exposed to dose levels of safrole of 0.163 and 1.655 mg/subject given orally (corresponding to 0.0027 mg/kg bw and 0.0276 mg/ kg bw for a 60 kg person) (Benedetti *et al.*, 1977). The metabolites to be excreted were calculated as the sum of 2′,3′-dihydroxysafrole, 1,2-dihydroxy-4-allylbenzene, 1′-hydroxysafrole glucuronide and 1′-oxosafrole formed in liver and 3′-hydroxysafrole formed in the liver and kidney.

The results of the Monte Carlo simulations show that at a dose of 0.163 mg/subject (Figure 3.6A), the PBBK model predicted percentage excreted as metabolites and the actual percentage excreted in human experiments are comparable. The simulation shows that at 6 h upon oral administration of 0.163 mg/subject, the 5th, median and 95th percentile of the predicted percentage of the dose excreted as safrole metabolites were 37%, 59% and 78% of the dose, respectively, whereas the experimental value reported in the literature amounted to 55 $\pm$ 15.6% (Benedetti *et al.*, 1977). Upon 12 h of exposure, the reported value is 86 $\pm$ 1.3% of the dose and the predicted percentage of 5th, median, and 95th percentile were 46%, 68% and 84% of the dose, respectively.

At a dose of 1.655 mg/subject (Figure 3.6B), upon 6 h of exposure the percentage of the dose excreted as safrole metabolites is reported to be 70% (standard deviation not given) (Benedetti *et al.*, 1977), whereas the predicted values of the simulation were 37%, 59%, and 77% of the dose for the 5th, median and 95th percentile, respectively.

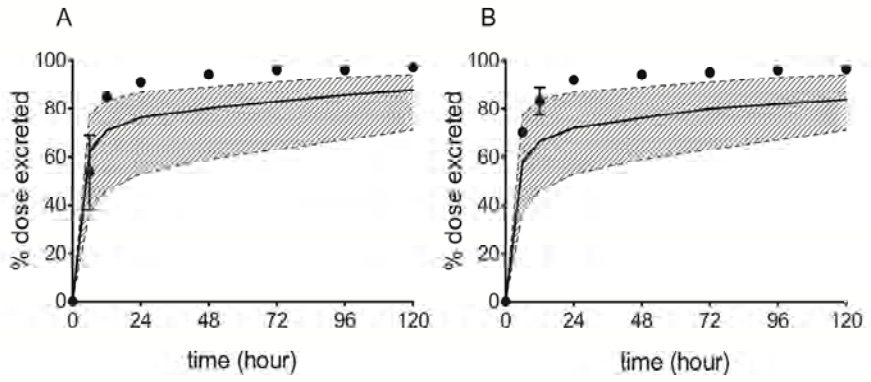

Figure 3.6. PBBK model and Monte Carlo simulation based predictions for interindividual variability in urinary safrole metabolite excretion (expressed as percentage of the dose administered) as compared to reported values (mean $\pm$ SD) (•) (Benedetti *et al.*, 1977) at oral dose levels of (A) 0.163 mg/subject (corresponding to 0.0027 mg/kg bw for a 60 kg person) and (B) 1.655 mg/subject (corresponding to 0.0276 mg/kg bw for a 60 kg person in the PBBK model). The solid line corresponds to the predicted median value and the dashed lines to the 5th, and 95th percentiles. Note that for several reported data points, the standard deviation is so small that they are not visible.

Model simulation of the concentration of safrole in the liver and blood and the concentration of 1′-hydroxysafrole in the liver

In a next step, the PBBK model was used to make predictions for the time- and dose-dependent bioactivation and detoxification of safrole in humans. Figure 3.7 presents the time-dependent predictions for the concentration of safrole in human blood and liver and the concentration of 1′-hydroxysafrole in human liver upon oral exposure to 0.005 mg safrole/kg bw, which is the estimated dietary human intake reported by the SCF (2002). At a dose of 0.005 mg/kg bw, the maximum liver concentrations of safrole and 1′-hydroxysafrole were predicted to be achieved within less than 0.5 h upon dosing. Although safrole and 1′-hydroxysafrole concentrations in the liver are low after 12 h, full conversion is not yet reached at this time point. Only after 120 h a more complete conversion is achieved, with 88% of the dose being metabolized to the different microsomal metabolites of safrole and almost full conversion of the 1′-hydroxysafrole formed. The PBBK model-predicted levels of the different safrole metabolites formed after 120 h are displayed in Table 3.4. The major metabolites are 1,2-dihydroxy-4-allylbenzene and 1′-hydroxysafrole, which are predicted to amount to ~38 and ~36% of the dose, respectively. 1′-Hydroxysafrole was mostly converted to 1′-oxosafrole at a dose 0.005 mg/kg bw (10.8 nmol/kg bw, corresponding to 35% of the dose, and to 98% of the 1′-hydroxysafrole formed), whereas formation of the ultimate carcinogenic metabolite 1′-sulfooxysafrole was predicted to amount to 0.04% of the dose and to 0.1% of the amount of 1′-hydroxysafrole formed. Formation of 1′-hydroxysafrole glucuronide was predicted to amount to 0.8% of the dose and to 2.0% the amount of 1′-hydroxysafrole formed.

Table 3.4. PBBK model predicted quantities of metabolites formed during 120 h upon an oral dose of 0.005 mg/kg bw (equivalent to 31 nmol/kg bw)

Metabolite	Amount formed (nmol/kg bw)[a]	Percentage of the dose[a]	Percentage of 1′-hydroxysafrole
Microsomal conversion of safrole			
2′,3′-dihydroxysafrole	3 (2, 7)	11 (6, 23)	
1′-hydroxysafrole	11 (5, 17)	36 (16, 55)	
3′-hydroxysafrole	1 (0.6, 1.0)	3 (2, 4)	
1,2-dihydroxy-4-allylbenzene	12 (6, 17)	38 (19, 56)	
Conversion of 1′-hydroxysafrole			
1′-hydroxysafrole glucuronide	0.2 (0.06, 0.6)	0.8 (0.2, 2.0)	2.0
1′-oxosafrole	10.8 (5, 16)	35 (16, 53)	98
1′-sulfooxysafrole	0.01 (0.0027, 0.04)	0.04 (0.01, 0.13)	0.1

[a] Values between the parentheses represents the 5th and 95th percentiles.

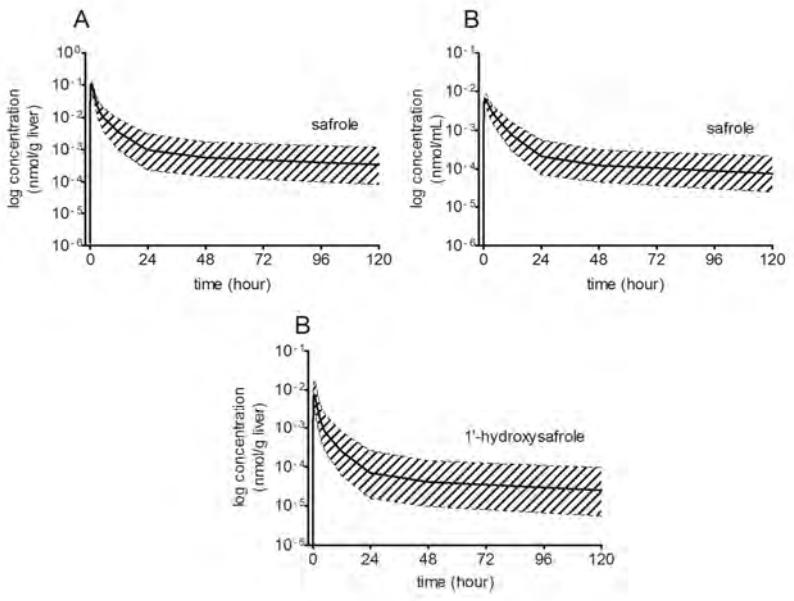

Figure 3.7. Monte Carlo simulation for the time-dependent concentration of safrole in A) liver and B) blood and C) time-dependent concentration of 1′-hydroxysafrole in the liver, upon oral exposure to 0.005 mg/kg bw safrole. No experimental data were available for comparison. The solid line indicates the predicted median value and the dashed lines the 5th and 95th percentile values.

The PBBK model-based predictions for the percentage of safrole metabolized to the various microsomal metabolites at increasing oral doses of safrole are presented in Figure 3.8. At a dose of 0.005 mg/kg bw the percentage of safrole metabolized to 2′,3′-dihydroxysafrole, 1′-hydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene was, respectively, 11, 36, 3, and 38% of the dose. When the dose was increased to 100 mg/kg bw, approximately 8, 21, 4, and 39% of the dose was predicted to be metabolized to 2′,3′-dihydroxysafrole, 1′-hydroxysafrole, 3′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene, respectively. These results reveal that 1,2-dihydroxy-4-allylbenzene is the major metabolite at low but also at high dose levels. When the dose increases the relative formation of 2′,3′-dihydroxysafrole and 1′-hydroxysafrole decreased only to a limited extent from 11 and 36% of the dose at 0.005 mg/kg bw to approximately 8 and 21% of the dose at 100 mg/kg bw, whereas the percentage formation of 1,2-dihydroxy-4-allylbenzene and 3′-hydroxysafrole tended to increase to a limited extent from values amounting to, respectively, 38 and 3% of the dose at 0.005 mg/kg bw to values of 39 and 4% of the dose at 100 mg/kg bw/day. Overall the results reveal that for safrole a dose-dependent shift in microsomal conversion of safrole leading to a relative increase in bioactivation at higher dose levels, is not observed. This can be explained by the fact that for microsomal conversion of safrole no saturation of an important metabolic route occurs in a dose range from 0.005 to 100 mg/kg bw/day.

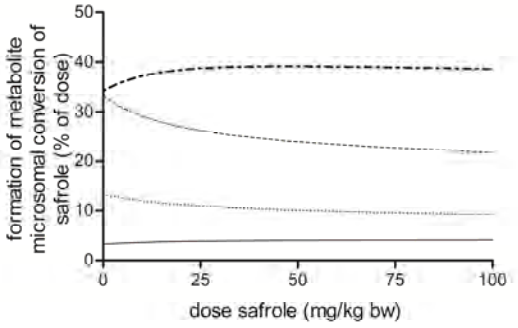

Figure 3.8. PBBK model based predicted dose-dependent changes in overall formation of microsomal safrole metabolites in all organs. The lines correspond to 2′,3′-dihydroxysafrole (••••), 1′-hydroxysafrole (— —), 3′-hydroxysafrole (——), and 1,2-dihydroxy-4-allylbenzene (—•—).

Model simulations of the formation of different metabolites of safrole and 1′-hydroxysafrole and comparison of those metabolites between humans and male rats

To obtain insight in possible species differences in metabolic activation and detoxification of safrole between humans and male rats, the results obtained with the PBBK model for safrole in humans were compared with those obtained by the previously defined PBBK model for safrole in male rats (Martati *et al.*, 2011). First, a comparison is made with respect to the possible species differences in the formation of various microsomal metabolites of safrole and in the formation of 1′-hydroxysafrole metabolites. This comparison was made at a dose level of 0.005 mg/kg bw, which is a dose level reflecting realistic daily human intakes. The second comparison focuses on the formation of 1′-sulfooxysafrole in human and male rat liver at dose levels varying from high doses representing dose levels causing liver tumors in long-term rodent bioassays, down to dose levels reflecting realistic human exposure.

Results from a Monte Carlo simulation of the formation of microsomal metabolites of safrole and of the conversion of 1′-hydroxysafrole and comparison of those metabolites between humans and rats are presented in Figure 3.9. Differences between humans and male rats mainly occur in the formation of 1′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene (Figure 3.9A) with the median formation of 1′-hydroxysafrole being fourfold higher in humans and the median formation 1,2-dihydroxy-4-allylbenzene being twofold lower in humans. The median formation of 2′,3′-dihydroxysafrole is 1.3- fold higher in humans than in male rats. The median formation of 3′-hydroxysafrole is twofold lower in humans than in male rats (Figure 3.9A). In humans, 1′-hydroxysafrole is mostly oxidized to 1′-oxosafrole (Figure 3.9B), which is due to the relative high efficiency for oxidation of 1′-hydroxysafrole in comparison with glucuronidation and sulfonation. Predicted formation of 1′-oxosafrole in humans is 98% of the 1′-hydroxysafrole formed while in rats this metabolite only accounts for 0.3% of the 1′-hydroxysafrole formed (Martati *et al.*, 2011). In male rats, 1′-hydroxysafrole is mostly converted to 1′-hydroxysafrole glucuronide corresponding to 99.5% of the 1′-hydroxysafrole formed (Martati *et al.*, 2011), a value that is predicted to amount to only 2% in human liver. Formation of the ultimate carcinogen 1′-sulfooxysafrole in humans was predicted to range between 0.128% (95^{th} percentile) and

0.01% (5^{th} percentile) of the dose with the median being fivefold higher in human than in rat liver (Figure 3.9B).

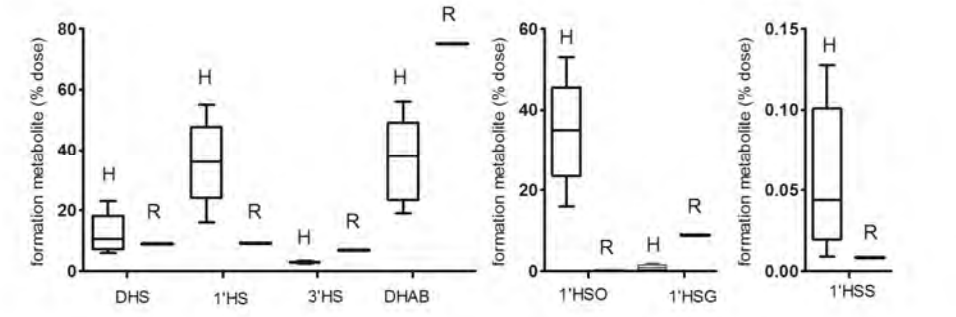

Figure 3.9. PBBK model and Monte Carlo simulation based prediction of (A) the formation of microsomal conversion of safrole in humans (indicated with H) and male rat (indicated with R) liver following an oral dose of 0.005 mg/kg bw safrole and (B) the cumulative levels of metabolites formed from 1′-hydroxysafrole in humans (indicated with H) and male rat liver (indicated with R) at this oral dose. Results of the Monte Carlo simulation obtained with the PBBK model for humans were presented as box-plots. The bottom and top of the boxes represent the 25^{th} and 75^{th} percentile, respectively and the the middle of the box is the 50^{th} percentile (the median). The ends of the whiskers attached to the box represent the the 5^{th} and 95^{th} percentile. For the rat model only the average predicted formation of different metabolites is displayed.

To see the possible dose-dependent changes in the formation of 1′-sulfooxysafrole in different species, the dose-dependent predicted formation of 1′-sulfooxysafrole in humans was compared with that predicted for rats. The predicted formation of 1′-sulfooxysafrole from a dose as low as 10^{-6} mg/kg bw to a dose as high as 100 mg/kg bw is presented in Figure 3.10. From these results it can be derived that the formation of 1′-sulfooxysafrole in human and rat liver is predicted to be linear up to doses as high as the benchmark dose (BMD_{10}) that gives 10% extra cancer incidence (Filipsson *et al*., 2003) amounting to 5-39 mg/kg bw (calculated based on data from Boberg *et al.* (1983) and Miller *et al.* (1983)) when plotted on a log-log scale (Figure 3.10) as well as on linear scale (data not shown). Over the whole dose range the median formation of 1′-sulfooxysafrole is three to fivefold higher in human than in rat liver, with the 5^{th} percentile being equal to the formation of 1′-sulfooxysafrole in rat liver and the 95^{th} percentile being 12- to 19-fold higher.

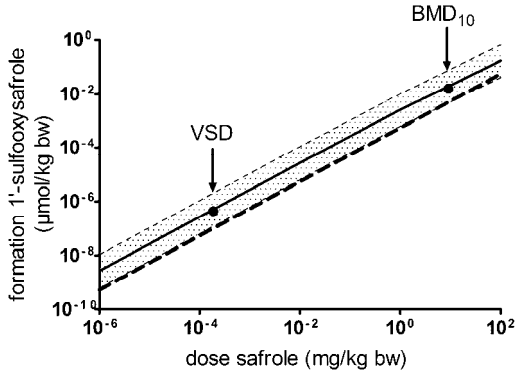

Figure 3.10. PBBK model-predicted dose-dependent formation of 1′- sulfooxysafrole in the liver of human, corresponding to the median or 50th percentile (———), the 5th and 95th percentiles (– –) and rat (— —). Note that the lines of the 5th percentile of 1′-sulfooxysafrole formation in humans and the mean of those in rats overlap.

Comparison of PBBK model-based predictions for safrole with the PBBK model-based predictions for the related alkenylbenzenes estragole and methyleugenol in humans

With the present PBBK model for safrole and the PBBK models for the related alkenylbenzenes estragole and methyleugenol previously developed (Al-Subeihi *et al.*, 2012; Punt *et al.*, 2009), comparisons can be made for the dose-dependent formation of the proximate carcinogenic 1′-hydroxy metabolites and also of the 1′-hydroxy glucuronide, 1′-oxo, and 1′-sulfooxy metabolites. Figure 3.11 presents the formation of these metabolites for the three alkenylbenzenes as predicted by the respective human PBBK models. Figure 3.11A shows that the predicted formation of 1′-hydroxysafrole is 12-fold lower than formation of 1′-hydroxyestragole and fivefold higher than formation of 1′-hydroxymethyleugenol. Figure 3.11B shows that the predicted formation of 1′-hydroxy glucuronide is very similar for the three alkenylbenzenes. Figure 3.11C shows that the predicted formation of 1′-oxosafrole is approximately twofold lower than formation of 1′-oxoestragole and 10-fold higher than formation of 1′-hydroxymethyleugenol. Figure 3.11D reveals that the formation of the unstable reactive 1′-sulfooxy metabolite in the human liver

is predicted to vary less than fivefold for the three alkenylbenzenes, being highest for estragole. The data obtained also reveal that for all three alkenylbenzenes the formation of the ultimate carcinogenic 1′-sulfooxy metabolite increases linearly with the dose up to dose levels of at least 10 mg/kg bw and that the overall conversion of safrole is slower than that of estragole and methyleugenol taking about 120 h instead of 24 h for full conversion.

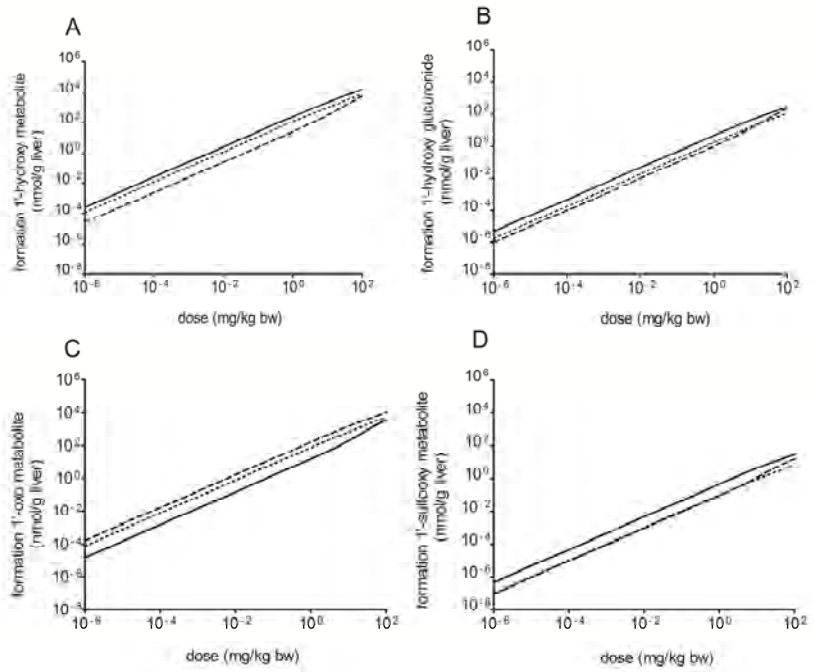

Figure 3.11. PBBK model based prediction of the dose-dependent formation of (A) the 1′-hydroxy metabolite, (B) the 1′-hydroxy glucuronide metabolite, (C) the 1′-oxo metabolite, and (D) the 1′-sulfooxy metabolite of estragole (———), methyleugenol (— — —), and safrole (…..) in pooled human liver after 120 h. The human PBBK models used for estragole and methyleugenol were previously described (Al-Subeihi *et al.*, 2012; Punt *et al.*, 2009).

Discussion

In the present study, a PBBK model was developed to provide possibilities for the evaluation of the relative extent of bioactivation and detoxification of safrole in humans at dose levels relevant for human dietary intake.

The performance of the developed PBBK model was evaluated by comparison of the predicted levels of urinary metabolite formation with the literature reported levels of the total safrole metabolites detected in the urine of humans exposed to two different oral doses of safrole (Benedetti *et al.*, 1977). The model predicted the time-dependent formation of safrole urinary metabolites reasonably well at the two dose levels of 0.0027 mg/kg bw for a 60 kg person (corresponding to 0.163 mg/subject) and 0.0276 mg/kg bw for a 60 kg person (corresponding to 1.655 mg/subject). The PBBK model-based prediction for the formation of safrole urinary metabolites at these dose levels amounted to 88% of the dose, whereas the percentage experimentally reported amounted to 97 and 96% of the dose, respectively. Based on results of a Monte Carlo simulation the differences observed were found to be partly due to uncertainty and variability in rates of formation of different metabolites and/or in other parameters shown in the sensitivity analysis to influence the modeling outcomes to the largest extent.

Benedetti *et al.* (1977) revealed that treatment of the urine of humans exposed to 1.655 mg/subject with β-glucuronidase resulted in extracted safrole metabolites at levels that were lower than those obtained for the urine of a rat exposed to safrole. This correlates with the finding of the present study that the glucuronidation of 1′-hydroxysafrole is a minor pathway in humans when compared with rats, leaving the possibility for other conversions of 1′-hydroxysafrole in humans. Although the extent of oxidation of 1′-hydroxysafrole to 1′-oxosafrole could not be evaluated against human in vivo data, the present study reveals that in human liver oxidation of 1′-hydroxysafrole is the main detoxification route of 1′-hydroxysafrole and this is in line with what was observed before for detoxification of 1′-hydroxyestragole and 1′-hydroxymethyleugenol in human liver (Al-Subeihi *et al.*, 2012; Punt *et al.*, 2009).

The PBBK model-predictions revealed that the relative formation of the various metabolites of microsomal conversion of safrole as well as the formation of the different 1′-hydroxysafrole metabolites is hardly changing at dose levels up to 100 mg/kg bw in humans (Figure 3.8). The PBBK model for safrole previously developed for male rats revealed similar results, showing also no dose-dependent effects on the relative extent of bioactivation and detoxification (Martati *et al.*, 2011). The PBBK model-predictions on species differences and interindividual human variation in formation of 1′-sulfooxysafrole can be used to derive so-called chemical-specific adjustment factors (CSAFs), which allow to evaluate the appropriateness of the default uncertainty factors used in risk assessment for species differences and interindividual human variation in kinetics (Meek *et al.*, 2003). Comparison of the median 1′-sulfooxysafrole up to dose levels of 100 mg/kg bw between rats and humans, revealed that species differences are moderate, amounting to only three- to fivefold. Species-dependent differences in the bioactivation of safrole to 1′-sulfooxysafrole are thus in line with the default factor of 4 generally assumed to reflect interspecies variation in kinetics (assuming that the default factor of 10 can be divided into a factor of 4 for kinetics and 2.5 for dynamics) (IPCS, 2010). Monte Carlo simulations were performed to evaluate the effect of uncertainty and variability on the predicted formation of 1′-sulfooxysafrole in the liver. Based on these results a CSAF for interindividual differences in kinetics can be calculated by dividing the parameter estimate at the 95^{th} percentile by the parameter estimate at the median (IPCS, 2005). The CSAF obtained in this way for the predicted formation of 1′-sulfooxysafrole is 3.9. Comparison of the CSAF with the default uncertainty factor of 3.16 for human variability in biokinetic reveals that the default uncertainty factor adequately protects 95% of the population.

Different approaches worldwide exist to assess the risk of compounds that are both genotoxic and carcinogenic. Numerical estimates of the risk associated with human exposure might be derived by extrapolation of carcinogenicity data obtained in animals at high to low dose levels relevant for the human situation. Linear extrapolation is the simplest form by which such extrapolations below the available experimental data set can be performed (COC, 2004). This approach is, however, much debated, because it is not known whether linear extrapolation actually reflects the underlying biological processes. In

addition, it is argued that species differences are not taken into account in this approach (EFSA, 2005). The results of the PBBK models for rats (Martati *et al.*, 2011) and humans obtained in the present study, however, indicate that the kinetic data do not provide a reason to argue against such a linear extrapolation from the rat tumor data to the human situation. As illustrated in Figure 3.10, the dose-dependent formation of 1′-sulfooxysafrole in the liver of rat and human is linear from doses as high as the BMD_{10} down to dose levels as low as the virtual safe dose (VSD) that gives one additional cancer risk in a million upon life time exposure) amounting to 0.046-0.39 µg/kg bw when plotted on a log-log scale as done in Figure 3.10 and also when plotted on a linear scale (Figure not shown). Because the BMD_{10} appears to be located within the linear part of the curves and the rat and human curves do not differ substantially, the PBBK results of the present study support that possible non-linear kinetics and species differences in kinetics should not be used as an argument against using a linear low dose cancer risk extrapolation from animal data to the low dose human situation. This illustrates that PBBK models provide a useful tool in risk assessment of food-borne chemicals when evaluating human risks.

The 1′-hydroxy metabolites of the alkenylbenzenes safrole, methyleugenol, and estragole and their sulphate conjugates have been shown to form DNA adducts in vivo and in vitro in a dose-dependent manner. It is of interest to compare the results for the PBBK predicted formation of the ultimate carcinogenic metabolites of the three alkenylbenzenes with their relative potency for DNA adduct formation in vitro and in vivo and even with their BMD_{10} values for tumor formation. In human liver, the differences in the level of the predicted formation of the ultimate carcinogenic 1′-sulfooxy metabolite formed from the three alkenylbenzenes were less than fourfold, the formation being highest for estragole. For rat liver, we have previously reported similar (fivefold) differences in bioactivation of the three alkenylbenzenes to their ultimate carcinogenic 1′-sulfooxy metabolites (Martati *et al.*, 2011). In HepG2 cells exposed to the alkenylbenzenes, the dose-dependent formation of DNA adducts decreased in the order methyleugenol > estragole > safrole but varied at most 3.5-fold. Furthermore, the estimated calculated $BMDL_{10}$ values of safrole, methyleugenol, and estragole, amount to approximately 3-29 mg/kg bw/day (calculated using BMD software based on the data from Boberg *et al.* (1983) and Miller *et al.* (1983)), 22-32 mg/kg

bw (Rietjens *et al.*, 2008), and 9-33 mg/kg bw/day (Rietjens *et al.*, 2010), respectively. These results together indicate that in both rats and humans the level of bioactivation of the three alkenylbenzenes is comparable, which is in line with their comparable carcinogenic potential.

Funding

Directorate General of Higher Education Ministry of National Education Republic of Indonesia.

Acknowledgments

The authors would like to thank Dr. Yiannis Fiamegos for synthesis of 1′-hydroxysafrole, Dr. Judy Bolton and the Department of Medicinal Chemistry and Pharmacognosy College of Pharmacy The University of Illinois at Chicago for providing synthesized 1,2-dihydroxy-4-allylbenzene and Ans Soffers for checking all the calculations.

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CHAPTER 4

Identification of malabaricone C as an important spice constituent inhibiting safrole bioactivation and physiology-based biokinetic modeling of its possible in vivo effect

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submitted to Toxicology Letter

Abstract

Safrole, a natural constituent of mace, causes liver tumors in rodent bioassays at high dose levels. This is ascribed to formation of DNA reactive 1′-sulfooxysafrole. The present paper identifies malabaricone C as a mace constituent able to inhibit safrole DNA adduct formation at the level of sulfotransferase (SULT)-mediated bioactivation. The SULT inhibition was incorporated into the physiologically based biokinetic (PBBK) rat and human models for safrole to investigate the possible in vivo effect of co-administration of safrole and malabaricone C-containing mace extract. At a dose of 50 mg/kg body weight (bw), assuming 100 or 1% uptake of malabaricone C and a ratio of malabaricone C-containing mace extract to safrole similar to what is found in mace, inhibition of 1′-sulfooxysafrole formation by malabaricone C-containing mace extract for rats and humans amounts to 90 and 100% or 61 and 91%, respectively. Altogether these data suggest a potential reduction of the cancer risk when safrole exposure occurs via mace within its normal food matrix compared to what is observed upon exposure to safrole as a pure compound.

Introduction

Myristica fragrans Houtt. belongs to the family Myristicaceae and is the source for two important spices produced from different parts of the plant: nutmeg which originates from the seeds and mace which is made from the dried aril cover of the seeds. Mace and its essential oils are most widely used as flavouring agents for sauces, meat and other food products. However, mace as well as nutmeg are known to contain so-called alkenylbenzenes including safrole (Archer, 1988) which has been recognized to be DNA reactive and carcinogenic in rodent bioassays when given at high dose levels (Daimon *et al*., 1997; Daimon *et al*., 1998). In addition, the habit of chewing betel quid in the Taiwanese population was reported to result in safrole concentrations up to 420 µM in saliva during chewing and has been linked to the increased incidence of oral squamous cell carcinoma (Hwang *et al*., 1992; Ko *et al*., 1995). Furthermore, safrole-like DNA adducts were detected with high frequency in oral tissues from oral cancer patients (Chen e*t al*., 1999).

The DNA reactive and hepatocarcinogenic effects of alkenylbenzenes have been linked to their bioactivation via the formation of a 1′-hydroxy metabolite catalyzed by cytochromes P450 followed by sulfation resulting the ultimate reactive 1′-sulfooxy metabolite that forms covalent adducts with cellular macromolecules including DNA, RNA and proteins (Borchert *et al*., 1973a; Wislocki *et al*., 1977). Figure 4.1 schematically presents the pathway for bioactivation of safrole and the nature of the DNA adducts formed. The DNA adducts found in hepatic tissue of rats exposed to safrole were identified as N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine (S-3′-N^2-dGuo), N^2-(safrol-1′-yl)-2′-deoxyguanosine (S-1′-N^2-dGuo) and N^6-(*trans*-isosafrol-3′-yl)-2′-deoxyadenosine (S-3′-N^6-dAdo) (Randerath *et al*., 1984). The S-3′-N^2-dGuo and S-1′-N^2-dGuo were the major adducts found in patients with oral squamous cell carcinoma resulting from the habit of betel quid chewing (Chen *et al*., 1999).

The Committee of Experts on Flavouring Substances (CEFS) of the Council of Europe evaluated safrole and concluded that safrole is a weak hepatocarcinogen and that efforts should be made to reduce its consumption through foods and beverages as much as possible

(Council of Europe, 1997). Safrole and sassafras oil were banned as food additives in 1960 by the U.S. Food and Drug Administration (FDA). The Scientific Committee on Food (SCF) concluded that safrole is genotoxic and carcinogenic and that reduction in exposure and restriction in the use levels are indicated (SCF, 2002). Human exposure to safrole is mainly via consumption of herbs and spices mostly nutmeg, mace and their essential oils (SCF, 2002). One may argue that risk assessment resulting from consumption of herbs and spices that contain safrole should take into account the possible modulating effects of other compounds present in these herbs or spices on the cytochrome P450 and/or sulfotransferase (SULT)-catalyzed bioactivation of safrole. Some spices were found to inhibit cytochrome P450 2C9, one of the cytochrome isoforms found to be involved in the metabolism of safrole to 1′-hydroxysafrole (Jeurissen *et al*., 2004; Kimura *et al*., 2010; Ueng *et al*., 2004). Furthermore for the related alkenylbenzene estragole present in basil, it was recently demonstrated that a methanolic basil extract and its isolated constituent nevadensin inhibited the sulfation and DNA adduct formation of the proximate carcinogenic 1′-hydroxyestragole metabolite in studies using rat and human S9 protein, the hepatoma cell line HepG2 and/or rat hepatocytes (Alhusainy *et al*., 2010; Jeurissen *et al*., 2008). Therefore, the objective of the present study was to investigate whether safrole containing spices contain ingredients that may inhibit the SULT mediated bioactivation of safrole. Mace was chosen as the model spice of interest because it contains significant levels of safrole (0.43-1.99%) (Archer, 1988). Upon identifying a major mace ingredient able to inhibit SULT mediated bioactivation and DNA adduct formation of 1′-hydroxysafrole in in vitro model systems, the possible effect of combined in vivo exposure was investigated by incorporating the kinetics for SULT inhibition into our recently developed physiologically based biokinetic (PBBK) models for formation of 1′-sulfooxysafrole in the rat and human liver.

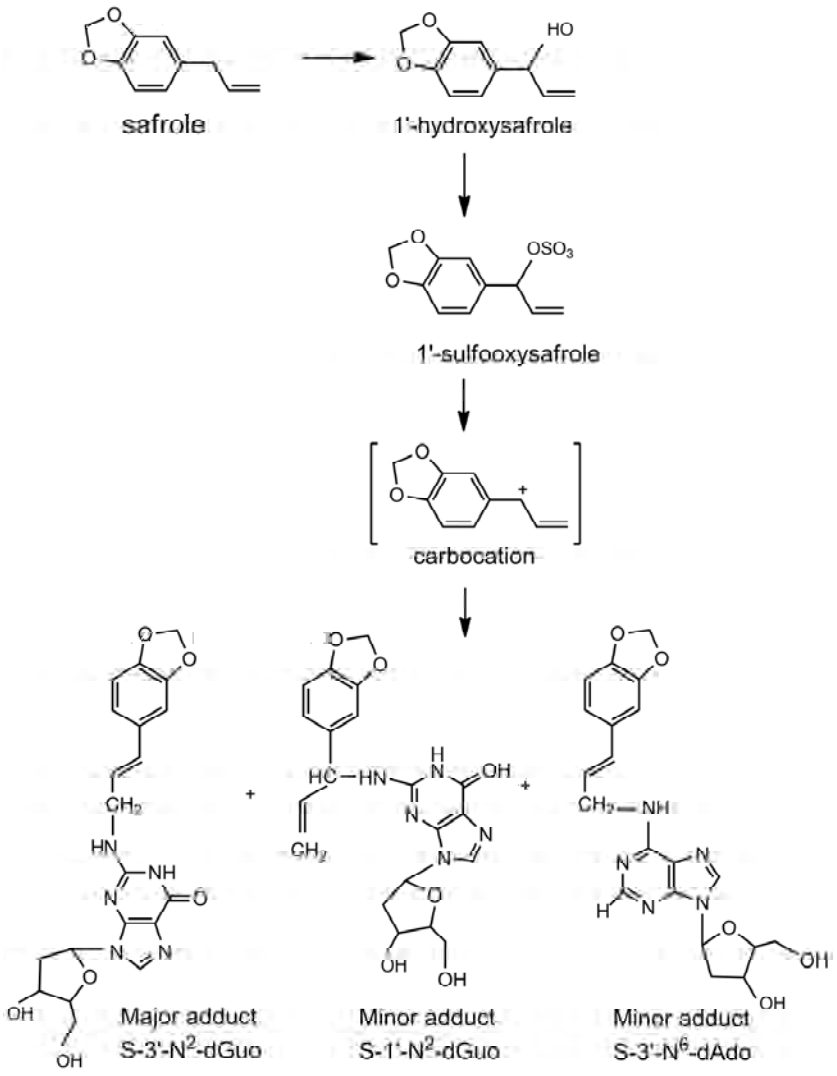

Figure 4.1. Bioactivation pathways of safrole and the structure of DNA adducts as identified by (Chung *et al*., 2008; Daimon *et al*., 1998; Randerath *et al*., 1984; Phillips *et al*., 1981). S-3′-N^2-dGuo = N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine, S-1′-N^2-dGuo = N^2-(safrol-1′-yl)-2′-deoxyguanosine and S-3′-N^6-dAdo= N^6-(*trans*-isosafrol-3-yl)-2′-deoxyadenosine.

Materials and methods

Caution : The following chemicals are hazardous and should be handled carefully: 1′-hydroxysafrole and 1′-acetoxysafrole.

Powder dried mace was obtained from a local supermarket. Pooled human and male rat liver S9 (Sprague-Dawley) were purchased from BD Gentest (Worburn, U.S.). The HepG2 cell line was purchased from American type culture collection (Manassas, Virginia). Culture medium DMEM/F12 (L-glutamine, 15 mM HEPES) and phosphate buffer saline (PBS, pH 7.4) were purchased from GIBCO (Paisley, UK). The chemical compounds were obtained with highest purity available. 7-Hydroxycoumarin (7HC), 7-hydroxycoumarin sulphate potassium salt (7HCS), 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), ammonium bicarbonate, Tris(hydroxymethyl)aminomethane, uridine 5′-diphosphoglucuronic acid (UDPGA), 3′-phosphoadenosine-5′-phosphosulphate (PAPS), 2′-deoxyguanosine (2′dGuo), pentachlorophenol (PCP), glutathione reduced (GSH), phosphodiesterase I from *Crotalus adamenteus* (venom phosphodiesterase, VPDE), phosphodiesterase II from bovine spleen (spleen phosphodiesterase, SPDE), alkaline phosphatase from *Escherichia coli*, and nuclease P1 from *Penicillium citrinum* were obtained from Sigma-Aldrich (Steinheim, Germany). Oxidized β-nicotinamide adenine dinucleotide phosphate (NAD^+) was obtained from Roche Diagnostics (Mannheim, Germany). Methanol and ethanol (pro analysis), hydrochloric acid, acetic acid glacial, formic acid, ethylenediaminetetraacetic acid (EDTA), sodium phosphate, zinc sulphate ($ZnSO_4.7H_2O$), pyridine and trifluoroacetic acid (Uvasol) were purchased from VWR (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) and dichloromethane were purchased from Acros Organic (New Jersey, U.S.). Acetic anhydride was supplied by J.T. Baker (Deventer, The Netherlands). Chromatography grade acetonitrile and methanol were purchased from Biosolve BV (Valkenswaard, The Netherlands). 1′-Hydroxysafrole was synthesized and purified as described previously (Jeurissen *et al*., 2004; Martati *et al*., 2011). 1′-Acetoxysafrole was synthesized from 1′-hydroxysafrole as described previously for synthesis of 1′-acetoxyestragole from the related alkenylbenzenes estragole (Borchert *et al*., 1973b; Punt *et al*., 2007).

Preparation of mace extract

A methanolic mace extract was prepared as previously described for preparation of methanolic extracts from basil (Alhusainy *et al.*, 2010). The extraction yield was approximately 32% (w/w). The mace extract obtained was subsequently diluted with methanol, to a concentration of 150 mg/mL. Methanolic mace extract and its stock of 150 mg/mL were kept at -20°C until usage.

Fractionation of mace extract

Mace extract was fractionated using preparative HPLC using a Waters 600 controller liquid chromatography system with Waters 2996 PDA-detector (Waters, Milford, MA). To this end, 50 µL of a solution of 150 mg methanolic mace extract/mL was injected onto a preparative column Sunfire C18 (5 µm, 10 x 250 mm). The mobile phase was made with ultrapure water containing 0.1 % (v/v) acetic acid and methanol. The flow rate was 4 mL/min and a gradient was applied from 0 % to 100% methanol in 80 minutes, and kept at 100% for 10 minutes, after which the column was re-equilibrated with the initial condition for 10 minutes. A total of 37 fractions were collected. Methanol was subsequently evaporated under nitrogen stream. Prior to freeze drying, the fractions were kept overnight at -80°C. The freeze drying was performed using an Alpha 1-2 LDplus freeze dryer (Salmenkipp BV, The Netherlands) at approximately 0.63 mbar. The samples thus obtained were dissolved in 100 µL methanol and kept at -80°C until further analysis for SULT inhibiting activity using the SULT activity assay.

Measurement of SULT activity

The effect of the methanolic mace extract and its isolated freeze dried fractions on SULT activity was investigated using the substrate 7HC and performed as previously described (Alhusainy *et al.*, 2010). For quantification of 7HCS in the incubations for SULT activity, aliquots of 3.5 µL of the incubation mixtures were analyzed on a Waters UPLC/DAD system consisting of a Waters Acquity binary solvent manager, sample manager and photodiode array detector (Waters, Milford, MA), equipped with a Waters Acquity UPLC BEH C18 column (1.7 µm, 2.1 x 50 mm). Quantification of 7HCS was achieved by

comparison of the peak area of the 7HCS in the chromatograms obtained at wavelength of 280 nm to the calibration curve of a commercially available of reference compound.

Determination of IC_{50}

The IC_{50} for the mace extract mediated inhibition of SULT was determined in incubation with rat or pooled human S9 based on incubation for SULT activity performed as described above, with 25 µM 7HC and concentration of mace extract ranging from 0.01 to750 mg/L The IC_{50} values of mace extract were determined by fitting the data to the standard enzyme hyperbolic inhibition equation: $y = V_{max} * (1 - (x /(IC_{50} + x))) + Y2$, using the Life Science Workbench (LSW) data analysis toolbox (version 1.1.1, MDL information system, San Leandro, CA). In this equation, Y represents the percentage of inhibition of 7HCS formation compared to control, Y2 is the lowest value of Y, V_{max} refers to the velocity observed in the absence of mace extract and x is the concentration of mace extract.

Determination of the inhibition constant

The inhibition constant for SULT inhibition was determined in incubations with rat or pooled human S9 by the mace extract based on incubations for SULT activity performed as described above, with concentrations of 7HC increasing from 0 to 25 µM in the absence or presence of mace extract at the concentration close to the IC_{50} for the respective S9 preparation. The type of inhibition by mace extract was determined with SigmaPlot Enzyme Kinetics Module (Systat Software, Inc, San Jose, CA). The initial velocities observed in the presence or absence of mace extract were automatically fitted to the equations and the best fitting inhibition model was selected. Mace extract appeared to exhibit a mixed-type inhibition of SULT catalyzed formation of 7HCS with rat and human liver S9 protein (see Results section). The inhibition constant (K_i) and the factor alpha describing this type of inhibition were obtained. The factor alpha reveals the effect of inhibitor on the binding of the enzyme for its substrate as well as the effect of the substrate on the binding of the enzyme for its inhibitor (Copeland, 2000).

^{1} H-NMR analysis

The fraction showing the most potent SULT-inhibiting activity was analyzed by ^{1}H-NMR to identify the compound(s) present. To this end, the methanol was evaporated under a nitrogen flow, followed by freeze drying and the sample was dissolved in 200 µL deuterated methanol in a 3 mm NMR tube (Bruker match system). ^{1}H-NMR analysis was performed using a Bruker Avance III 600 MHz NMR spectrometer (Bruker Ettlingen, Germany) equipped with a cryoprobe. A Noesygppr1d pulse sequence with a 3 s delay, 0.1 s mixing time and a 1.8 s acquisition time were used (18,028 Hz sweep width, 64 K data points). Spectra were obtained at 300 K. Resonances were reported relative to deuterated methanol at 3.34 ppm. In addition 2D ^{1}H-^{1}H NMR data were acquired (COSY and TOCSY) as well as ^{1}H-^{13}C HSQC data.

Cytotoxicity test

Cytotoxicity of 1′-hydroxysafrole and the mace extract towards HepG2 cells was determined using the MTT test (Jeurissen *et al.*, 2008). The optical density of each well was measured with a plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength 562 and 620 nm for the colour intensity and background, respectively. The cell viability was defined as the ratio between the absorbance of exposed cells as compared to the absorbance for untreated controls.

Formation of S-3′-N^2-dGuo in HepG2 cells exposed to 1′-hydroxysafrole and mace extract

HepG2 cells were grown in culture medium (DMEM/F12 with glutamine containing 10% foetal calf serum) in a 75 mL flask. Cell confluence of 90% was required prior to exposure. At 90% confluence, the growth medium was removed and the cells were exposed to fresh medium containing the test compounds. 1′-Hydroxysafrole was added from a 1000 times concentrated stock solution in DMSO to give a final concentration of 100 µM. Mace extract (final concentrations of 0, 5, 25, and 100 mg/L) was added from 1000 times concentrated stock solutions in methanol. After exposure for 14 hours at 37°C and 5% CO_2, the cells were scrapped in PBS (pH 7.4) and collected in a 1.5 mL Eppendorf tube and centrifuged at

1500 rpm for 5 min. The pellets were stored at -20°C until DNA isolation. Isolation of DNA from HepG2 cells exposed to the test compounds was performed as described previously (Paini *et al*., 2010).

Synthesis of S-3′-N^2-dGuo

S-3′-N^2-dGuo was prepared as described previously for synthesis of the N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine (E-3′-N^2-dGuo) adduct of the related alkenylbenzene estragole (Punt *et al*., 2007). To obtain pure synthesized S-3′-N^2-dGuo, the reaction mixture was purified using HPLC-UV (Waters 600 controller liquid chromatography system) equipped with an Alltima C18 column of 5 µm, 150 x 4.6 mm (Grace Alltech, Breda, The Netherlands). The flow rate was set at 1 mL/min. The gradient was made with ultrapure water and acetonitrile. A gradient was applied from 20% to 26% acetonitrile over 40 min after which the percentage of acetonitrile was increased to 100% in 2 min and kept at 100% for 1 min. The initial condition was reached within 2 min and kept for 15 min. Under these conditions, the peak corresponding to S-3′-N^2-dGuo eluted at a retention time of 22.6 min. Collected fractions were pooled and the acetonitrile was evaporated under nitrogen. Freeze drying was performed after the collected fractions were kept overnight at -80°C.

Quantification DNA adducts by LC-MS

Quantification of S-3′-N^2-dGuo was performed using LC-ECI-MS/MS on a Perkin Elmer 200 series HPLC System (Perkin Elmer, Waltham, MA) coupled to an API 3000 system (Applied Biosystem, Foster city, CA) as previously described for detection of E-3′-N^2-dGuo (Paini *et al.*, 2012; Punt *et al.*, 2007). To this end, an aliquot of 10 µL was injected on an Agilent Zorbax Extend-C18 column, 2.1×50 mm, 3.5 Micron 80 Å (Basel, Switzerland), with a Zorbax guard column. The gradient was made with ultrapure water containing 0.1% (v/v) formic acid and acetonitrile. The flow rate was set at 0.3 mL/min and a gradient was applied from 20% to 70% acetonitrile over 8 min, after which the percentage of acetonitrile was brought to 100% in 1 min and kept at 100% acetonitrile for 2 min. The initial condition was restored in 1 min and the column was equilibrated at this condition for 8 min. Under these conditions S-3′-N^2-dGuo eluted at 2.7 min. The mass spectrometric analysis was done

in positive ion mode. The ion spray voltage was set at 4 kV and the ion source temperature was set at 250°C. The nebulizer gas (air), curtain (nitrogen) and collision gas (nitrogen) were set to 15, 10 and 5 psi respectively. A divert valve was used in order to discard the eluent during the first 2 min and the gradient after elution of the peak. Sample analysis was carried out using the selected reaction monitoring (SRM) mode and characteristic transitions were recorded. The most intense transition was used as quantifier and subsequent transitions as qualifiers, for S-3′-N^2-dGuo these transitions were as follows: 428 → 312 m/z (quantifier), 428 → 164 m/z (qualifier), 428 → 161 m/z (qualifier).

Effect of mace extract on 1′-hydroxysafrole glucuronidation and oxidation

To determine the inhibiting potency of mace extract on glucuronidation and oxidation of 1′-hydroxysafrole, incubations were performed with human or rat liver S9 protein (for glucuronidation) or human or rat liver microsomes (for oxidation). The incubations were performed as described previously (Martati *et al*., 2011; Martati *et al*., 2012) in the absence or presence of 100 mg/L of mace extract added from a 200 times concentrated stock solution of mace extract in methanol. The type of inhibition by mace extract was determined with SigmaPlot Enzyme Kinetics Module (Systat Software, Inc, San Jose, CA). The initial velocities observed in the presence or absences of mace extract were automatically fitted to the equations and the best fitting inhibition model was selected and the values of K_i (the inhibition constants) are also obtained.

Safrole PBBK models

The PBBK models used to predict the effect of the SULT inhibition by mace extract on safrole bioactivation in vivo were based on the PBBK models previously described for safrole in rats (Martati *et al*., 2011) and humans (Martati *et al*., 2012). The values of the apparent maximum velocity ($V_{max(app)}$) for formation of microsomal safrole metabolites and of 1′-oxosafrole were scaled to the liver using a microsomal protein yield of 32 mg/g liver (Barter *et al*., 2007). The $V_{max(app)}$ values for sulfation and glucuronidation of 1′-hydroxysafrole were scaled to liver using an S9 protein yield of 143 mg/g liver (Medinsky *et al*., 1994).

Safrole was assumed to be absorbed directly from the gastrointestinal tract to the liver following first order kinetics with a default absorption constant set at 1.0 h^{-1}. In this study, the inhibition of SULT-mediated formation of 1′-sulfooxysafrole by the mace extract was included in the PBBK model with a function for mixed inhibition. The resulting modified Michaelis-Menten equation for the formation of 1′-sulfooxysafrole that was incorporated in the model was as follows:

$$dAM_{HSS}/dt=V_{max,HSS}/(1+I/(alpha*K_{i,HSS}))*CL_{HS}/PL_{HS}/(K_{m,HSS}*(1+I/K_{i,HSS})/\ (1+I*(alpha*K_{i,HSS}))+(CL_{HS}/PL_{HS})$$

In this equation, $K_{i,HSS}$ and alpha*$K_{i,HSS}$ are the dissociation constants of the enzyme-inhibitor complex and of enzyme-substrate-inhibitor complex for the sulfation reaction, respectively. $K_{i,HSS}$ and alpha*$K_{i,HSS}$ were expressed as mg/L. $V_{max,HSS}$ and $K_{m,HSS}$ are the values representing the maximum rate and Michaelis-Menten constant for sulfonation of 1′-hydroxysafrole, expressed as nmol/min/mg S9 protein and µM, respectively. CL_{HS} is the concentration of 1′-hydroxysafrole in the liver (µM), P_{LHS} is the liver/blood partition coefficient of 1′-hydroxysafrole.

In incubation with rat tissue fractions, mace extract also exhibited competitive inhibition of oxidation of 1′-hydroxysafrole and non-competitive inhibition of glucuronidation of 1′-hydoxysafrole. These reactions were not inhibited in incubations with human liver fractions. To this end, only in the rat PBBK model the Michaelis-Menten equations describing oxidation and glucuronidation were modified as follows:

$$dAM_{HOS}/dt = V_{max,HOS} *CL_{HS}/PL_{HS}/((K_{m,HOS})*(1+(I/K_{i,HOS})+CL_{HS}/PL_{HS})$$

$$dAM_{HSG}/dt= V_{max,HSG}/(1+I/K_{i,HSG})*CL_{HS}/PL_{HS}/(K_{m,HSG}+CL_{HS}/PL_{HS})$$

In this equations, $K_{i,HOS}$ and $K_{i,HSG}$ represent the dissociation constants of the enzyme-inhibitor complex for oxidation and glucuronidation. Both $K_{i,HOS}$ and $K_{i,HSG}$ were expressed

as mg/L. $V_{max,x}$ and $K_{m,x}$ are the values representing the maximum rate and Michaelis-Menten constant, respectively, for oxidation (x=HOS) and glucuronidation (x=HSG) of 1′-hydroxysafrole (expressed as nmol/min/mg protein and µM, respectively). CL_{HS} is the concentration of 1′-hydroxysafrole in the liver (µM), P_{LHS} is the liver/blood partition coefficient of 1′-hydroxysafrole.

In the PBBK model, mace extract was assumed to undergo the same fate as safrole in the liver as described by the following the equation: [I] = CL * f, where [I] is the concentration of mace extract (mg/L), CL is the concentration of safrole in the liver (µmol/L), and f is the ratio mace extract (mg) and safrole (µmol). To see the possible difference in kinetics and bioavailability of safrole and mace extract the model was run with a variable ratio between these compounds (expressed as mg/µmol). The ratio was varied from 0 (no mace extract) to 44 (representing an amount of mace extract (expressed as mg) that is 44 times higher than safrole (expressed as µmol). From this simulation formation of 1′-sulfooxysafrole, 1′-oxosafrole and 1′-hydroxysafrole glucuronide were calculated after 72 h (for rat model) (Martati *et al*., 2011) or 120 h (for human model) (Martati *et al*., 2012) representing time points at which safrole conversion appeared to be complete. The dose of safrole used for the PBBK modeling was 0.005 mg safrole/kg body weight (bw) which is the dose representing human daily intake (SCF, 2002).

Statistical Analysis

A *t*-test was performed using Excel (Microsoft Office 2010) to evaluate whether the differences between treatments were statistically significant. Variances equality were tested with Levene's test (SPSS 15.0 for Windows, SPSS Inc. Chicago, IL).

Results

Inhibition of SULT activity by mace extract

Figure 4.2A shows the inhibition of SULT activity by increasing concentrations of the methanolic mace extract. Increasing concentrations of the mace extract inhibited SULT activity in a dose-dependent manner. For comparison the figure also shows the effect of 25 µM of the well-known SULT inhibitor PCP. Incubations without co-factor PAPS did not

show any formation of 7HCS, whereas, control incubations with methanol or DMSO elicited no inhibition of SULT mediated 7HCS formation (data not shown).

SULT inhibition by different fractions of mace extract

Figure 4.2B shows the SULT inhibiting potency of each of the 37 mace fractions collected upon HPLC fractionation of the methanolic mace extract. The fractions with the most potent SULT inhibiting activity were fractions 23 (with approximately 91% inhibition), followed by fraction 18 (88%) and fraction 19 (57%). The most potent mace fraction, fraction number 23, was analyzed using HPLC revealing that it contained one major compound (chromatogram not shown) and subsequently analyzed using ^{1}H-NMR and ^{13}C-NMR in order to identify this compound.

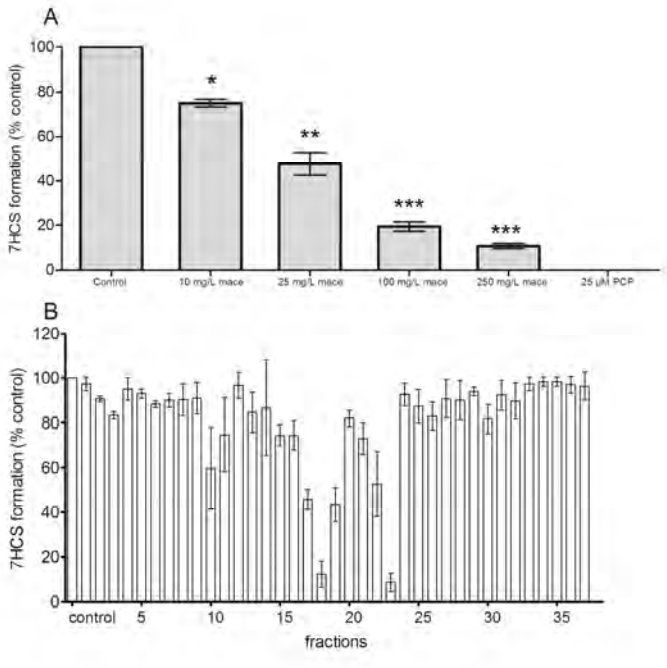

Figure 4.2. Inhibition by mace extract of the SULT mediated conversion of 7HC to 7HCS by pooled rat liver S9 protein in the presence of (A) an increasing concentration of mace extract or 25 µM PCP or (B) collected mace fractions. Data points represent mean ± SD of three independent measurements. An asterisk (*) indicates a significant inhibition compared to the incubation without inhibitor (* $p < 0.05$, ** $p<0.01$, *** $p< 0.001$).

Identification of a potent inhibitor in mace extract

^{1}H-NMR and ^{13}C-NMR characteristics of the compound present in fraction 23 perfectly matched the molecule no 7 (malabaricone C) reported by (Pham *et al*., 2000). Figure 4.3 presents the structure of this newly identified SULT inhibitor from mace.

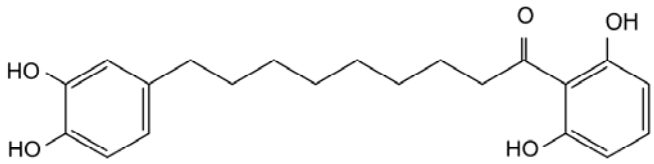

Figure 4.3. Chemical structure of malabaricone C, the newly identified SULT inhibitor in mace extract

Effect of mace extract on the kinetics of SULT activity

Inhibition of SULT activity by mace extract indicated that the inhibition was dose dependent (Figure 4.4). The IC_{50} values of mace extract derived from these curves are 41 and 3.2 mg/L for rat and human liver S9 protein, respectively. For determination of the K_i values for the SULT inhibition by mace extract, 7HC concentration-dependent SULT activity was measured in the absence (uninhibited) or presence of 50 mg/L or 3 mg/L of mace extract for respectively rat and pooled human S9. Figure 4.5 presents the formation of 7HCS by pooled rat and human liver S9 versus the 7HC substrate concentration in the presence and absence of mace extract. The apparent K_{max} and V_{max} values derived from the curve obtained in the absence of inhibitor are presented in Table 4.1. The catalytic efficiency ($V_{max}/K_{m)}$ for SULT-dependent formation of 7HCS was about three times higher for male rat liver S9 protein than that for human liver S9 protein. In rats, the apparent V_{max} for sulfation was two times lower and the K_m increased three times in the presence of 50 mg/L mace extract. Whereas in humans, in the presence of 3 mg/L of mace extract the apparent V_{max} was 1.7 times lower than the V_{max} obtained in the absence of mace extract and the K_m values was 2.3 times higher. These results indicate that both V_{max} as well as K_m are

affected to a similar extent upon addition of the mace extract pointing at a mixed-type inhibition for both rat and human SULT activity in the liver by mace extract. The K_i values for inhibition of SULT activity were 8.3 and 1.0 mg/L for rat and human liver S9, whereas the alpha*K_i derived from these curves amounted to 41.5 and 4 mg/L.

Table 4.1. Kinetic parameters of SULT-dependent formation of 7HCS by pooled rat and human liver S9 protein in the absence and presence of mace extract.

	Without mace extract		With mace extract[1]			
Species	Apparent V_{max} (pmol/min/mg S9 protein	Apparent K_m of 7HC (μM)	Apparent V_{max} (pmol/min/mg S9 protein	Apparent K_m of 7HC (μM)	K_i (mg/L)	alpha*K_i (mg/L)
Rat	1341 ± 53	3.0 ± 0.4	622 ± 49	8.8 ± 1.7	8.3	41.5
Human	301 ± 14	2.2 ± 0.4	174 ± 11	5.0 ± 0.9	1.0	4.0

[1] Concentration of mace extract used were 50 and 3 mg/L for, respectively, rat and pooled human S9.
Data represent mean ± SD of three replications (for rats) and five replications (for humans).
K_i is a dissociation constant of the enzyme-inhibitor complex.
alpha*K_i is a dissociation constant of the enzyme-substrate-inhibitor complex.

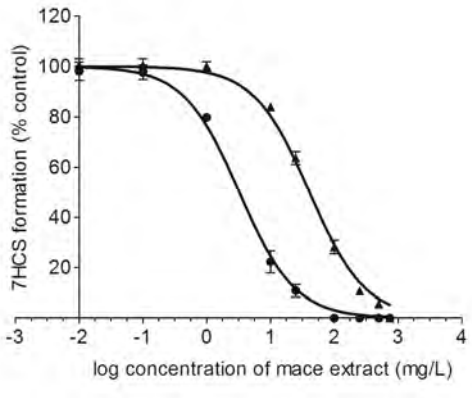

Figure 4.4. Mace extract concentration dependent SULT inhibition in incubations with male rat liver S9 protein (▲) and pooled human liver S9 protein (●).

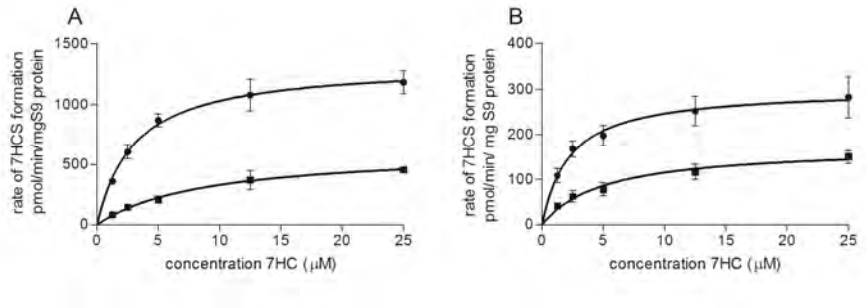

Figure 4.5. 7HC concentration dependent formation of 7HCS in the absence (•) or presence (■) of mace extract (50 mg/L for rat liver S9 or 3 mg/L for pooled human liver S9) in incubation with (A) male rat liver S9 protein and (B) pooled human liver S9 protein.

Inhibition of DNA adduct formation by mace extract in HepG2 cells exposed to 1′-hydroxysafrole

Figure 4.6 presents the formation S-3′-N^2-dGuo detected in HepG2 cells exposed to 100 μM 1′-hydroxysafrole for 14 h in the absence and presence of increasing concentrations of mace extract. The mace extract did not display cytotoxicity up to concentrations of at least 100 mg/L which was the highest concentration tested. The results revealed that mace extract inhibited formation of S-3′-N^2-dGuo in 1′-hydroxsafrole exposed HepG2 cells an effect that increased with the concentration of the mace extract and was statistically significant at concentration of 25 mg/L of mace extract and above.

Effect of mace extract on glucuronidation and oxidation of 1′-hydroxysafrole

Table 4.2 presents the apparent K_m and V_{max} for glucuronidation and oxidation of 1′-hydroxysafrole in the absence and presence of 100 mg/L mace extract with pooled rat and human liver S9 or microsomal protein. In rats, the apparent V_{max} for oxidation was 1.3 times higher and the K_m increased 2.8 times in the presence of 100 mg/L of mace extract.

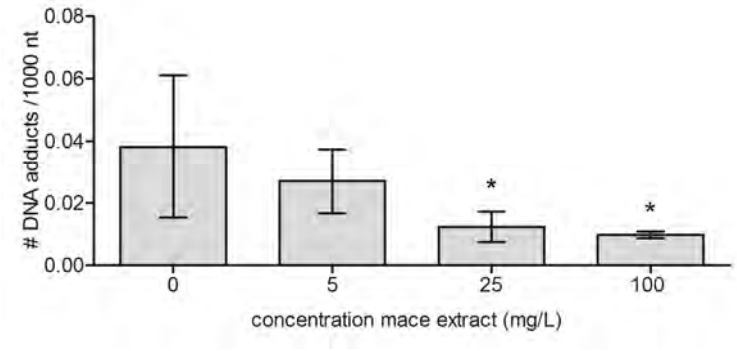

Figure 4.6. S-3′-N^2-dGuo formation in HepG2 cells exposed to 100 μM 1′-hydroxysafrole in the absence or presence of an increasing concentration of mace extract. Data points represent mean ± SD. An asterik (*) indicates significant inhibition ($P < 0.05$).

Whereas for glucuronidation, the V_{max} is 1.5 times lower and the K_m increased 2.5 times. These results point at competitive inhibition type for oxidation of 1′-hydroxysafrole and non-competitive type inhibition for glucuronidation of 1′-hydroxysafrole. Based on data presented in Table 4.2, the K_i values were determined to amount to 140 mg/L and 86 mg/L for oxidation and glucuronidation of 1′-hydroxysafrole, respectively. In human, addition of 100 mg/L of mace extract to the incubation has no effect in glucuronidation and oxidation of 1′-hydroxysafrole because the apparent V_{max} and K_m were not significantly different compared with those values in the absence of 100 mg/L of mace extract.

PBBK-based prediction for conversion of 1′-hydroxysafrole in the presence of mace extract

The PBBK model for detoxification and bioactivation of safrole in both rat and human were modified to take the inhibition by malabaricone C-containing mace extract into account and allow investigation of possible in vivo effects on safrole bioactivation to 1′-sulfooxysafrole. PBBK model-based predictions were made at safrole dose levels of 0.005 mg/kg bw and 50 mg/kg bw (Figure 4.7). The dose level of 0.005 mg/kg bw was selected to take into account

realistic human dietary exposure levels and the dose of 50 mg/kg bw was chosen to include dose levels known to cause tumors in in vivo rodent studies. The PBBK model revealed that oral administration of a dose of safrole 0.005 mg/kg bw (SCF, 2002) together with increasing dose levels of mace extract result in a decrease in formation of the ultimate carcinogenic metabolite 1′-sulfooxysafrole. At a ratio between mace extract and safrole of 44 (mg/µmol) reflecting the level of these constituents in mace (as characterized by the extraction efficiency and the safrole content of mace quantified by HPLC analysis of the methanolic extracts, data not shown), the predicted inhibition of formation of 1′-sulfooxysafrole in rat and human liver were 4 and 49%, at a dose 0.005 mg/kg bw of safrole, respectively, assuming 100% uptake of mace extract and its constituent, including malabaricone C to follow the same kinetic profile as safrole. Figure 4.7B presents the predicted formation of 1′-sulfooxysafrole at a dose of safrole of 50 mg/kg bw. At a ratio of mace extract and safrole of 44, the inhibition of 1′-sulfooxysafrole formation for rat and human liver are 90 and 100% or 61 and 91% assuming 100 and 1% mace extract uptake, respectively.

Tables 4.2. Kinetic parameters for glucuronidation and oxidation of 1′-hydroxysafrole in incubations with pooled rat and human liver S9 or microsomal protein in the absence and presence of mace extract.

Species	Without mace extract		With 100 mg/L mace extract		
	Apparent V_{max} (nmol/min/mg S9 protein or microsomal protein)	Apparent K_m (µM)	Apparent V_{max} (nmol/min/mg S9 protein or microsomal protein)	Apparent K_m (µM)	K_i (mg/L)
Oxidation					
Rat	5.0 ± 0.4	1719 ± 293	6.7 ± 0.6	4791 ± 671	140
Human	8.0 ± 0.3	670 ± 75	7.5 ± 0.6	911 ± 202	
Glucuronidation					
Rat	17.6 ± 1.6	224 ± 56	11.7 ± 2.0	563 ± 198	86
Human	0.09 ± 0.02	537 ± 299	0.09 ± 0.01	565 ± 148	

Data represent mean ± SD of three replications.
K_i is dissociation constant of the enzyme-inhibitor complex

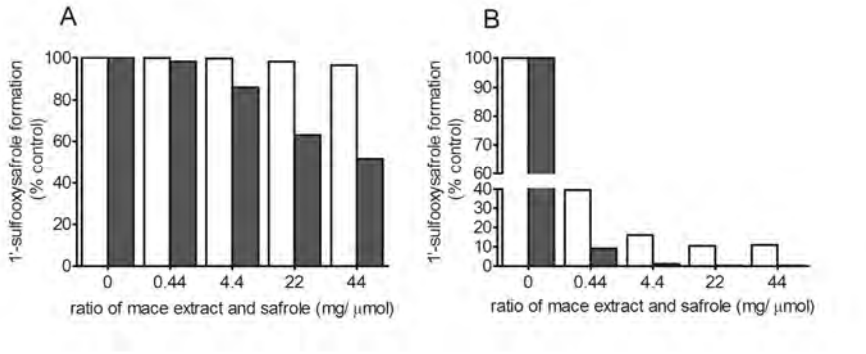

Figure 4.7. PBBK-based predictions for the dose-dependent effect of mace extract on 1′-sulfooxysafrole formation in the liver of rat (white bars) and human (black bars) at oral dose level of (A) 0.005 mg/kg bw and (B) 50 mg/kg.

Discussion

Safrole is an alkenylbenzene occurring in many spices. The compound was shown to be hepatocarcinogenic in rodents when administered at high dose levels as a pure compound (Borchert *et al*., 1973a; Borchert *et al*., 1973b; Daimon *et al*., 1997; Daimon *et al*., 1998). The aim of the present study was to investigate whether ingredients present in safrole containing herbs and spices, using mace as the model spice, can interact with the SULT mediated bioactivation of safrole pointing at a possible reduction in bioactivation when the compound would be dosed in its natural food matrix instead of as a pure compound. In a previous study, it was demonstrated that the flavonoid nevadensin, a constituent in basil, is a potent inhibitor of bioactivation of the related alkenylbenzene estragole at the level of the SULT-mediated conversion of the proximate carcinogenic 1′-hydroxy metabolite to the ultimate carcinogenic 1′-sulfooxy metabolite (Jeurissen *et al*., 2008; Alhusainy *et al*., 2010). The results of the present study reveal that mace also contains a potent SULT inhibitor which was identified as malabaricone C. The K_i for SULT inhibition by malabaricone C-containing mace extract was 8.3 and 1.0 mg/L for male rat and human liver fractions, respectively. Given that malabaricone C was not available as a pure compound only an estimate of the K_i for malabaricone C can be made. Mace contains 0.53% malabaricone C (Orabi *et al*., 1991). Taking 32% extraction yield for preparation of the

mace extract and assuming all malabaricone C to be extracted in mace extract, the content of malabaricone C in mace extract is 1.66%. Assuming that 50% of the inhibition observed with mace extract would be due to malabaricone C, these K_i values would amount to 0.19 and 0.023 µM malabaricone C. These K_i values would be in the same range as the K_i values determined previously for other SULT inhibitors in the diet like kaempferol and apigenin which K_i values of 0.6 µM and 0.7 µM were observed (Alhusainy *et al*., 2012). The K_i values are higher than the K_i value previously identified for nevadensin for which the K_i value amounted to 4 nM. The mechanism of inhibition of SULT activity by malabaricone C-containing mace extract in this present study was mixed type inhibition resulting an increased apparent K_m and a decreased apparent V_{max}. The alpha*K_i values obtained in the present study for malabaricone C-containing mace extract for rat liver and human were four to five times higher than the K_i. This indicates that the inhibitor has higher affinity for the free enzyme than for the enzyme-substrate complex. In a previous study, inhibition of SULT activity by different flavones as well as by nevadensin was found to be non-competitive (Alhusainy *et al*., 2010) and also a great volume of literature data supports the plausibility of non-competitive inhibition of sulfonation of various substrates by different flavonoids such as quercetin, genistein, daidzein, (+)-catechin, kaempferol, apigenin, and diadzein (Ghazali and Waring, 1999; Mesía-Vela and Kauffman, 2003; Walle *et al*., 1995). Coughtrie and Johnston (2001) showed that epicatechin gallate and epigallocatechin gallate showed uncompetitive inhibition to SULT1A1 but mixed type inhibition to SULT1A2-1A3. Recently, studies using *Salmonella typhimurium* TA100 strains with expression of human SULT revealed especially human SULT1A1 and SULT1C2 to be able to activate the 1′-hydroxymetabolite of the related alkenylbenzene methyleugenol to DNA reactive metabolites (Herrmann *et al*., 2012). The SULT enzymes responsible for conversion of 1′-hydroxysafrole to 1′-sulfooxysafrole remain to be established. To investigate whether the malabaricone C-containing mace extract would also be able to exert inhibition of the DNA adduct formation in an intact cell system, the effect of increasing concentrations of mace extract on safrole DNA adduct formation in cells from the human hepatoma cell line HepG2 exposed to 1′-hydroxysafrole was quantified (Brandon *et al*., 2003; Knasmüller *et al*., 1998). Exposure of HepG2 cells to 1′-hydroxysafrole in the presence of an increasing

concentration of malabaricone C-containing mace extract revealed a dose-dependent inhibition of S-3′-N^2-dGuo formation.

Furthermore, the kinetics for inhibition of SULT activity by malabaricone C-containing mace extract were integrated into the previously defined PBBK models for safrole bioactivation and detoxification in male rat and human liver (Martati, *et al*., 2011, Martati, *et al*., 2012). The modified PBBK model allowed prediction of bioactivation and detoxification of safrole in the rat and human liver when safrole would be dosed in the presence of mace extract. At a dose of safrole 50 mg/kg bw and ratio of 44 mg mace extract/μmol safrole, the predicted inhibition of formation of 1′-sulfooxysafrole in male rat and human liver were 90 and 100% and 61 and 91% assuming 100 and 1% uptake of malabaricone C, respectively. Thus formation of 1′-sulfooxysafrole is predicted to be inhibited although not completely blocked upon co-exposure to safrole and the newly identified SULT inhibitor present in mace, malabaricone C.

In conclusion, the present study identifies an important SULT inhibitor present in mace to be malabaricone C. This points at a possible matrix effect of combined exposure to safrole and malabaricone C. For the human liver samples, the SULT inhibition was shown to lead to the inhibition of the formation of the ultimate carcinogenic metabolite, without reducing the rate of detoxification of the proximate carcinogen 1′-hydroxysafrole via glucuronidation or oxidation. This indicate a potential reduction of the cancer risk when safrole intake via mace results from the food matrix containing malabaricone C or other SULT inhibitors presents in the regular daily diet (SCF, 2002) compared to what is observed upon exposure to safrole as a pure compound. Validation of the relevance of the interaction of this SULT inhibitor with safrole bioactivation in an *in vivo* study will provide further support for the hypothesis that this combination effect should be taken into account in risk assessment of alkenylbenzenes.

Acknowledgments

This work was supported by the Directorate General of Higher Education Ministry of National Education Republic of Indonesia through the “DIKTI Scholarship”. The authors would like to thank Dr. Yiannis Fiamegos for synthesis of 1′-hydroxysafrole.

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CHAPTER 5

Malabaricone C-containing mace extract inhibits safrole DNA adduct formation in the liver of orally exposed rats

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in preparation

Abstract

The present study investigated whether the malabaricone C-containing mace extract that was previously shown to be able to inhibit safrole DNA adduct formation in vitro can also inhibit safrole DNA adduct formation in vivo. To this end, mace extract and safrole were co-administered orally to Sprague-Dawley rats and the level of safrole DNA adduct formation in the liver was quantified and compared to the level of safrole DNA adducts detected in the liver of rats exposed to safrole only. Since the inhibition of safrole DNA adduct formation by malabaricone C-containing mace extract was previously shown to occur at the level of the sulfotransferase (SULT) -mediated bioactivation of 1′-hydroxysafrole to 1′-sulfooxysafrole, a third group of rats exposed to safrole together with the well-known SULT inhibitor pentachlorophenol (PCP) was also included. LC-ECI-MS/MS based quantification of DNA adduct levels in the liver of safrole exposed rats revealed a significant ($p<0.01$) 55% and 64% reduction of safrole DNA adduct formation when exposure to safrole occured in the presence of malabaricone-C containing mace extract or PCP, respectively. Given the role of DNA adduct formation in the hepatocarcinogenicity of safrole, the results of the present study suggest that the likelihood of bioactivation and subsequent adverse effects may be lower, when safrole is dosed in rodents bioassays within a relevant food matrix containing SULT inhibitors compared to dosing of pure safrole. Using the data obtained a refined risk assessment using the Margin of Exposure (MOE) approach was performed. The results obtained point at a potential reduction of the cancer risk resulting in increased MOE values and a lower priority for risk management when the carcinogenicity of safrole would have been tested within a relevant food matrix containing SULT inhibitors compared to what is observed in rodent bioassays upon exposure to pure safrole dosed by gavage.

Introduction

Some spices such as mace, nutmeg, star anise and black pepper contain the alkenylbenzene safrole, a compound that has been proven to be genotoxic and carcinogenic at high dose levels in rodent studies (Borchert *et al.*, 1973; Miller *et al.*, 1983; Wislocki *et al.*, 1977; Daimon *et al.*, 1998). Bioactivation of safrole is initiated by cytochromes P450 resulting in formation of the proximate carcinogenic metabolite 1′-hydroxysafrole followed by sulfonation mediated by sulfotransferases (SULTs) and resulting in formation of the ultimate carcinogenic metabolite 1′-sulfooxysafrole. 1′-Sulfooxysafrole forms covalent adducts with cellular macromolecules including DNA (Borchert *et al.*, 1973; 1980; Wislocki *et al.*, 1977). The role of 1′-sulfooxysafrole in the formation of DNA adducts in the liver of rat and mice dosed with safrole is supported by the observation that this DNA adduct formation can be inhibited by co-administration of safrole with pentachlorophenol (PCP), a known SULT inhibitor (Boberg *et al.*, 1983; Daimon *et al.*, 1997; Randerath *et al.*, 1984). The DNA adducts isolated from the liver of rats exposed to safrole were N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine (S-3′-N^2-dGuo), N^2-(safrol-1′-yl)-2′-deoxyguanosine (S-1′-N^2-dGuo) and N^6-(*trans*-isosafrol-3′-yl)-2′-deoxyadenosine (S-3′-N^6-dAdo) (Randerath *et al.*, 1984). DNA adducts identified in patients with oral squamous cell carcinoma resulting from the habit of betel quid chewing were S-3′-N^2-dGuo and S-1′-N^2-dGuo (Chen *et al.*, 1999).

In the European Union, the use of safrole as such in food has been banned since 2008, whereas in the U.S. it was already banned since 1960. As a result, the current daily exposure to safrole mainly results from the intake of safrole with spices or food products to which safrole-containing herbs or spices or their essential oils are added including for example cola and alcoholic beverages and sausages (Choong and Lin, 2001; Curró *et al.*, 1987; Siano *et al.*, 2003). This raises the question of whether rodent studies dosing safrole at high levels as a pure compound present an adequate basis for assessment of the risks associated with the intake of safrole in a complex food matrix.

In a previous study, we demonstrated that mace extract and especially its constituent malabaricone C inhibited safrole DNA adduct formation at the level of SULT-mediated

bioactivation and formation of S-3′-N^2-dGuo in 1′-hydroxysafrole exposed HepG2 cells (Martati *et al.*, 2012). A physiologically based biokinetic (PBBK) model analysis of safrole bioactivation and detoxification in rat, incorporating the SULT inhibition to predict the possible in vivo effect, predicted that administration of safrole at dose levels generally used in rodent cancer bioassays in the presence of malabaricone C-containing mace extract would significantly inhibit the 1′-sulfooxysafrole formation, thereby possibly reducing the cancer risk and influencing the risk assessment.

The aim of the present study was to investigate whether the inhibition by the ingredients present in mace extract of SULT activity and S-3′-N^2-dGuo adduct formation in incubations with male rat liver S9 fractions and in HepG2 cells (Martati *et al.*, 2011; Martati *et al.*, 2012) can also be demonstrated in vivo in a rat model. The results obtained were also used to make a refined risk assessment of safrole.

Materials and methods

Fine ground powdered mace was obtained from a local supermarket. 2′-Deoxyguanosine (2′dGuo), pentachlorophenol (PCP), phosphodiesterase I from *Crotalus adamenteus* (venom phosphodiesterase, VPDE), phosphodiesterase II from bovine spleen (spleen phosphodiesterase, SPDE), alkaline phosphatase from *Escherichia coli*, nuclease P1 from *Penicillium citrinum*, β-glucuronidase from *Helix pomatia* Type HP-2, and ethyl acetate were obtained from Sigma–Aldrich (Steinheim, Germany). Methanol and ethanol (pro analysis), hydrogen chloride, acetic acid glacial, formic acid, ethylenediaminetetraacetic acid (EDTA), sodium phosphate, sodium acetate, trifluoroacetic acid and $ZnSO_{4*}7H_2O$ were purchased from Merck (Darmstadt, Germany). Acetic anhydride was supplied by J.T. Baker (Deventer, The Netherlands). Pyridine was purchased from VWR (Darmstadt, Germany). Chromatography grade acetonitrile and methanol were purchased from Biosolve BV (Valkenswaard, The Netherlands). Phosphate buffered saline (PBS, pH 7.4) was purchased from GIBCO (Paisley, UK). 1′-Hydroxysafrole was synthesized and purified as described previously (Jeurissen *et al.*, 2004; Martati *et al.*, 2011). 1′-Acetoxysafrole was synthesized from 1′-hydroxysafrole as described previously for synthesis of 1′-

acetoxyestragole from 1′-hydroxyestragole for the related alkenylbenzene estragole (Borchert *et al.*, 1973; Punt *et al.*, 2007).

S-3′-N^2-dGuo was prepared as described previously for synthesis of the E-3′-N^2-dGuo adduct of the related alkenylbenzene estragole (Punt *et al.*, 2007). To obtain pure synthesized S-3′-N^2-dGuo, the reaction mixture was purified using HPLC-UV (Waters 600 controller liquid chromatography system) equipped with an Alltima C18 column of 5 µm, 150 x 4.6 mm (Grace Alltech, Breda, The Netherlands). The flow rate was set at 1 mL/min. The gradient was made with ultrapure water and acetonitrile. A gradient was applied from 20% to 26% acetonitrile over 40 min after which the percentage of acetonitrile was increased to 100% in 2 min and kept at 100% for 1 min. The initial condition was reached within 2 min and kept for 15 min. Under these conditions, the peak corresponding to S-3′-N^2-dGuo eluted at a retention time of 22.6 min. Collected fractions were pooled and the acetonitrile was evaporated under a stream of nitrogen. Freeze drying was performed after the collected fractions were kept overnight at -80°C. S-3′-N^2-dGuo was identified and quantified by LC-ESI-MS/MS as described below.

Animals

The animal study was performed in accordance with the Dutch Act on animal experimentation (Stb, 1977,67; Stb 1996, 565), revised February 5, 1997. The protocol of the study was approved by the ethical committee on animal experimentation of Wageningen University. Seven week old male Sprague-Dawley (SD) rats were obtained from Harlan Laboratories (The Netherlands). The rats were housed in type IV cages (three rats per cage) with Lignocel (Lignocel BK 8/15) bedding. Animals were kept in the standard animal rooms under conditions of controlled temperature (21$\pm$1°C), humidity (55$\pm$10%) and a 12 h light/dark schedule and were monitored daily. Animals were given free access to feed (Teklad 2914C pellet) and tap water during the whole study (from arrival at the animal facility until sacrifice). Following a 5-day acclimatization period, animals were housed individually in the metabolic cages 24 h before gavage and were kept in the metabolic cages up to time of sacrifice (48 h after gavage).

Pilot Study

The in vivo studies consisted of a range finding pilot study and a main study. For the pilot study, eight animals were divided into four groups of 2 animals per group. These groups were exposed as follows: 1) first group dosed once with 150 mg/kg bw of safrole only in corn oil, 2) second group dosed once with 300 mg /kg bw of safrole only in corn oil, 3) third group dosed once with 150 mg/kg bw of safrole together with 463 mg/kg bw of mace extract simultaneously in corn oil, and 4) fourth group dosed once with 300 mg/kg bw of safrole together with 926 mg/kg bw of mace extract simultaneously in corn oil. The ratio between safrole and mace extract was chosen based on PBBK model based predictions selecting a ratio that would result in significant SULT inhibition but would require a dose of mace extract below the dose level reported to be toxic (El Malti *et al*., 2009). For the pilot study further animal handling and analyses were performed as described below for the main study.

Main Study

For the main study, fifteen rats were divided into three groups of 5 rats per group and exposed as follows: 1) safrole only group dosed once with 150 mg/kg bw safrole only in corn oil, 2) safrole plus mace extract group dosed once with 150 mg/kg bw of safrole together with 463 mg/kg bw of mace extract dosed simultaneously in corn oil and 3) safrole plus SULT inhibitor control group dosed once with 150 mg/kg bw of safrole together with 11 mg/kg bw of PCP simultaneously in corn oil.

A control group receiving only feed and carrier/corn oil was not included in the pilot or main study because recent studies showed that exposure to only feed and carrier/corn oil did not result in alkenylbenzene DNA adduct levels above the detection limit (Paini *et al.*, 2012). Safrole and or mace extract were administrated simultaneously using corn oil as carrier by gavage with the volume of gavage not exceeding 2 mL/kg bw. Animals were sacrificed 48 h after gavage. Anesthesia was done with a mixture of isoflurane and oxygen by inhalation, blood was removed after which the liver was removed, weighed and stored at -80°C until further analysis. The liver was separated in the left liver lobe and the rest of the

tissue containing the right liver lobe. For the pilot and main study, urine was collected at 0, 6, 24, 30 and 48 h after gavage and stored at -80°C for further analysis.

Preparation of mace extract

A methanolic mace extract was prepared as previously described for preparation of methanolic extracts from basil (Alhusainy *et al.*, 2010). Briefly, 10 g of mace and 200 mL of a mixture of methanol, ultrapure water and acetic acid (80:19:1) were stirred for an hour at room temperature. The extraction was performed twice, each time with 200 mL of solvent mixture. The collected extraction mixtures were filtered using a folded filter (Schleicher and Schuell, 150 mm). The filtrate was pooled and concentrated by evaporation at 35°C on a rotary evaporating system (Heidolph Laborota 4000 efficient, Metrohm U.S.). The extraction yield was approximately 32%. The concentrated mace extract was kept at -20°C until usage.

Tissue DNA extraction and digestion

Prior to DNA extraction, tissue samples were homogenized mechanically using a cell homogenizer (B. Melsungen Germany). For the liver, to prepare one sample, 4 random 0.8 g samples were taken from the left liver lobe as well as 4 random 0.8 g samples from the right liver lobe. Each of the samples was minced with a surgical knife and subsequently mashed with a mortar and pestle. Then 8 mL cold phosphate buffered saline (PBS) were added and samples were transferred and homogenized mechanically on ice using a cell homogenizer. The homogenates were transferred to a 15 mL tube and centrifuged at 3000 g for 5 min (Sigma centrifuge, type 4K10, Germany). The pellet was dissolved in 300 µL remaining supernatant and then divided into two equal parts. Each part was transferred to a 2 mL Eppendorf tube to provide sample replicates for analysis. The pellet was extracted with a Get pure DNA Kit-Cell protocol to obtain DNA according the manual of the Kit. The DNA pellet was dissolved in 100 µL of nanopure water. The yield and purity of isolated DNA were determined using the Nanodrop technology by measuring the absorbance ratio A260/A280nm. DNA samples with an absorbance ratio of 1.8 to 2 were considered sufficiently pure. The DNA quantity per sample was calculated from the Nanodrop output in ng/mL using a molar extinction coefficient for double stranded DNA of

50 L mol^{-1} cm^{-1} (Sambrook and Russell, 2001). The DNA obtained was digested as previously described (Delatour *et al.*, 2008) to release S-3′-N^2-dGuo. In short, to 50 µg DNA in 100 µL water, 20 µL buffer P1 (300 mM sodium acetate, 1 mM $ZnSO_4$, pH 5.3), 11 µL SPDE solution (0.0004 U/µL in water) and 10 µL nuclease P1 (0.0005 µg/µL in water) were added and the mixture was incubated for 4 h at 37°C. Subsequently, 20 µL buffer PA (500 mM Tris-HCl, 1 mM EDTA, pH 8.0), 10 µL VPDE solution (0.00026 U/µL in water) and 2.6 µL alkaline phosphatase (0.764 U/µL in water) were added and the mixture was incubated for 3 h at 37°C. The DNA hydrolysates thus obtained were passed through a 5000 Nominal Molecular Weight Limit (NMWL) cut-off filter for removal of the enzymes and subsequently evaporated to dryness and reconstituted in 50 µL ultrapure water and stored at -20°C until further analysis by LC-ESI-MS/MS.

Urine analysis

Urine analysis was performed as previously described (Benedetti *et al.*, 1977). De-conjugation of conjugated metabolites in the urine was performed using β-glucuronidase/sulfatase. Briefly, frozen urine was thawed quickly at 37°C in a waterbath. A sample of 5 µL urine was mixed with 45 µL 0.2 M sodium acetate pH 5 and 10 µL of β-glucuronidase from *Helix pomatia* (activity of glucuronidase 198.455 U/mL and sulfatase 813 U/mL). The mixture was incubated at 37°C for 2 h. The hydrolysate was extracted with 250 µL ethyl acetate using vortexing and centrifugation at 15.000 rpm for 3 min. The extraction was repeated twice using 250 µL ethyl acetate each time. The extracts thus obtained were pooled and solvent was evaporated under a stream of nitrogen and the residue was subsequently dissolved in ultrapure water and stored at -20°C until HPLC analysis.

Quantification of DNA adduct formation

Quantification of S-3′-N^2-dGuo was performed using LC-ECI-MS/MS on a Perkin Elmer 200 series HPLC System (Perkin Elmer, Waltham, MA) coupled to an API 3000 system (Applied Biosystem, Foster city, CA) as previously described for detection of N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine (E-3′-N^2-dGuo) (Paini *et al.*, 2012; Punt *et al.*, 2007). To this end an aliquot of 10 µL was injected on an Agilent Zorbax Extend-C18 column,

2.1×50 mm, 3.5 Micron 80 Å (Basel, Switzerland), with a Zorbax guard column. The gradient was made with ultrapure water containing 0.1% (v/v) formic acid and acetonitrile. The flow rate was set at 0.3 mL/min and a gradient was applied from 20% to 70% acetonitrile over 8 min, after which the percentage of acetonitrile was brought to 100% in 1 min and kept at 100% acetonitrile for 2 min. The initial condition was restored in 1 min and the column was equilibrated at this condition for 8 min. Under these conditions S-3′-N^2-dGuo eluted at 2.7 min. The mass spectrometric analysis was done in positive ion mode. The ion spray voltage was set at 4 kV and the ion source temperature was set at 250°C. The nebulizer gas (air), curtain (nitrogen) and collision gas (nitrogen) were set to 15, 10 and 5 psi, respectively. A divert valve was used in order to discard the eluent during the first 2 min and the gradient after elution of the peak. Sample analysis was carried out using the selected reaction monitoring (SRM) mode and characteristic transitions were recorded. The most intense transition was used as quantifier and subsequent transitions as qualifiers, for S-3′-N^2-dGuo these transitions were as follows: 428 → 312 m/z (quantifier), 428 → 164 m/z (qualifier), 428 → 161 m/z (qualifier).

Identification and quantification of urinary metabolites

Identification and quantification of urinary metabolites obtained after enzymatic de-conjugation was performed on a UPLC (Ultra Performance Liquid Chromatography)-DAD (diode array) system consisting of a Waters (Milford, MA) Acquity binary solvent manager, sample manager and photodiode array detector. Aliquots of 3.5 µL were injected on the Waters Acquity UPLC BEH RP 18 column (1.7 µm, 2.1 x 50 mm). The eluent consisted of ultrapure water containing trifluoroacetic acid 0.1% (v/v) and acetonitrile. At a flow rate of 0.6 mL/min, a gradient was applied from 10% to 40% acetonitrile over 2.9 min after which the percentage of acetonitrile was increased to 100% over 0.2 min and kept at 100% for 0.2 min. Thereafter, the percentage of acetonitrile was reduced to its initial value in 0.1 min and kept at this level for 1.2 min. Detection and quantification of the urinary metabolites were performed at 280 nm. Quantification of 1′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene was achieved by comparison of the peak areas obtained at a wavelength of 280 nm to the calibration curves made using the corresponding synthesized reference compounds.

PBBK-model-based predictions for formation of 1′-sulfooxysafrole in the liver of rat in the presence or absence of mace extract

The safrole PBBK model defined previously for male rat (Martati *et al.*, 2011) as well as the PBBK model defined for male rat including the SULT inhibition by malabaricone C-containing mace extract (Martati *et al.*, 2012) were used to obtain insight in the effects of SULT inhibition by malabaricone C-containing mace extract on the formation of the ultimate carcinogenic metabolite, 1′-sulfooxysafrole, in the liver. PBBK model based predictions for safrole bioactivation and its inhibition by malabaricone C-containing mace extract were compared to the results obtained for inhibition of DNA adduct formation in the liver of rats exposed to safrole in the absence or presence of malabaricone C-containing mace extract in order to further validate the PBBK models previously defined.

Refined risk assessment for safrole

To estimate the possible consequences of the inhibition of safrole bioactivation and DNA adduct formation by malabaricone C-containing mace extract for the risk assesment of safrole the margin of exposure (MOE) approach was used (EFSA, 2005). The MOE is defined as the ratio between a point of departure derived from the tumor data, preferably the $BMDL_{10}$ (the lower confidence limit of the benchmark dose causing 10% extra tumor incidence) and the estimated daily intake (EDI). An MOE of 10,000 or higher is considered as low priority for risk management (EFSA, 2005).

For the refined risk assessment of safrole, an estimate was made of the tumor incidences that would be expected when safrole would have been tested in the presence of malabaricone C-containing mace extract as done in the present study. To this end the available data on the incidence of hepatocellular carcinoma in female mice administered safrole via the diet for 52 weeks were taken as the starting point (Miller *et al.*, 1983). The dose levels reported in the study reported by Miller *et al*. (Miller *et al*., 1983) in mg/kg diet were converted to dose levels in mg/kg bw/day assuming a daily feed intake of female mice of 0.13 kg/kg bw/day, and adjusting for 1) 3 doses a week converted to daily administration (values multiplied by 3/7), 2) duration of the exposure converted to life time exposure (doses times 52/86) and 3) the loss of the compound on standing in open containers at room

temperature which was reported to amount to 5%/day (Miller *et al*., 1983). First $BMDL_{10}$ values for safrole were calculated from this data set using the BMD software version 2.4. MOE values were calculated by dividing the $BMDL_{10}$ values obtained by the EDI for regular human daily intake.

In a subsequent step, the tumor incidences in the analysis were reduced by the percentage inhibition of safrole DNA adduct formation detected in the present study upon co-administration of safrole with the malabaricone C-containing mace extract. BMD analysis of these refined data for hepatocellular carcinoma incidence resulted in refined $BMDL_{10}$ and MOE values that reflect the possible consequences of testing safrole carcinogenicity in the presence of the malabaricone C-containing mace extract for the risk assessment.

Statistical analysis

A *t*-test using Excel (Microsoft Office 2010) was performed to evaluate whether the differences between the treatments were statistically significant. Variances equality were tested with Levene's version of the F tested with SPSS 15.0 for Windows (SPSS Inc. Chicago, IL, U.S.).

Results

Pilot Study: Inhibition of DNA adduct formation by mace extract in the liver of male rats exposed to safrole

In a dose-finding pilot study, safrole was given by oral gavage at a dose of 150 mg/kg bw or 300 mg/kg bw with or without mace extract. When safrole was dosed together with mace extract, a ratio of mace extract to safrole of 0.5 mg/µmol was used. At this ratio, the PBBK model predicted approximately 80% inhibition of 1′-sulfooxysafrole formation. The level of DNA adduct formation was measured in the liver, 48 h after gavage. This time point was chosen based on a similar study with the related alkenylbenzene estragole which showed that at 48 h following gavage DNA adducts were significantly higher than at 24 h after gavage (Paini *et al.*, 2012). Figure 5.1 shows the DNA adduct formation in the liver of rats dosed with 150 and 300 mg/kg bw safrole. Formation of DNA adducts in the liver of rats dosed with 300 mg/kg bw safrole was 2.3-fold higher than DNA adduct formation in the

livers of rats dosed with 150 mg/kg bw safrole. Co-administration of safrole with mace extract resulted in a significant ($p<0.05$) inhibition of the formation of DNA adducts by 38 and 20% at 150 and 300 mg/kg bw safrole respectively. Based on the results obtained, the dose level of safrole of 150 mg/kg bw resulted in detectable and significant formation of safrole DNA adducts and the largest inhibition by mace extract. Therefore, 150 mg/kg bw safrole dosed with or without mace extract at a ratio of mace extract to safrole of 0.5 mg/µmol, was selected for the main study.

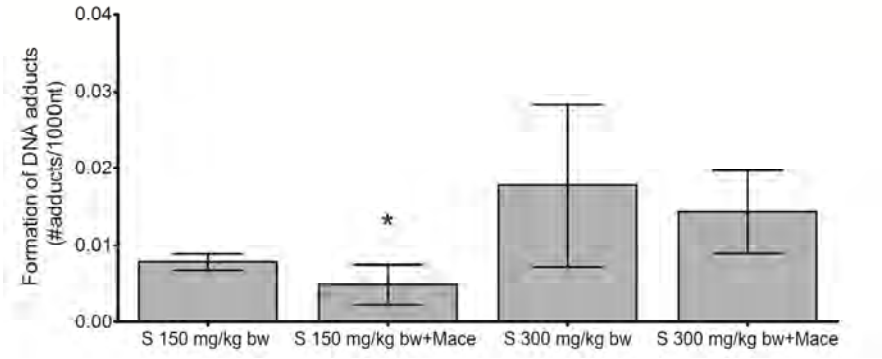

Figure 5.1. Formation of DNA adducts in the liver of SD rats treated with safrole at 150 mg/kg bw, safrole at 150 mg/kg bw + mace extract, safrole at 300 mg/kg bw or safrole at 300 mg/bw + mace extract. When safrole was dosed together with mace extract a ratio of mace extract to safrole of 0.5 mg/µmol was used. DNA adducts are expressed as number of adducts per 1000nt. Data represent the average and standard deviation of 2 rats. * = $p<0.05$

Main Study: Inhibition of DNA adduct formation by mace extract in the liver of male rats exposed to safrole

The main study consisted of three groups of 5 rats exposed to 1) safrole only, dosed with 150 mg/kg bw, 2) safrole and mace extract, dosed with 150 mg/kg bw safrole and 463 mg/kg bw mace extract, and 3) safrole and a positive control for SULT inhibition, dosed with 150 mg/kg bw + PCP 11 mg/kg bw. Figure 5.2 presents the levels of DNA adducts detected in the liver of rats exposed to safrole or safrole in the presence of mace extract or PCP. The formation of DNA adducts in the liver of rats exposed to 150 mg/kg bw safrole, 150 mg/kg bw safrole + 463 mg/kg bw mace extract or 150 mg/kg bw safrole +11 mg/kg bw PCP amounted to 0.0240 ± 0.0120, 0.0110 ± 0.0045 or 0.0084 ± 0.0018 adducts/1000nt,

respectively. The percentage inhibition of the formation of DNA adducts upon co-administration of safrole with mace extract or PCP was significant ($p<0.01$) and amounted to 55% and 64%, respectively.

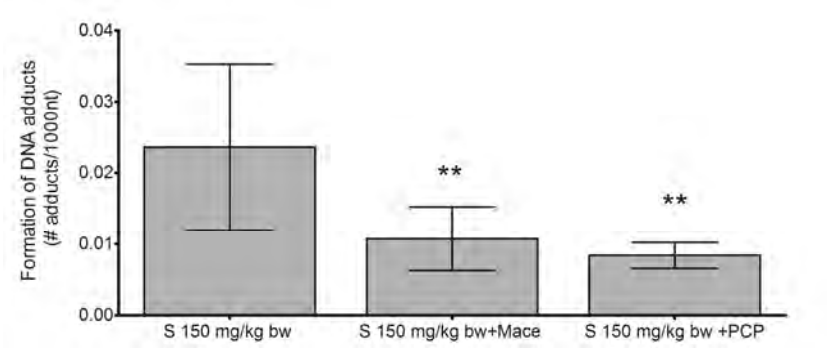

Figure 5.2. Formation of DNA adducts in the liver of rats treated with 150 mg/kg bw safrole, 150 mg/kg bw safrole + 463 mg/kg bw mace extract, or 150 mg/kg bw safrole + 11 mg/kg bw PCP. DNA adducts are expressed as number of adducts per 1000nt. Data represent the average and standard deviation of 5 rats (for the group treated with safrole + PCP) or 7 rats (for groups treated with safrole only and safrole + mace extract combining the data from the pilot and the main study) **= $p < 0.01$

Urinary metabolites of rats exposed to safrole and mace extract

In order to ascertain that co-exposure to safrole and mace extract or PCP did not reduce DNA adduct formation by affecting the bioavailability of safrole, urinary excretion of safrole metabolites in rats exposed to safrole with or without mace extract or PCP was quantified. Figure 5.3 presents the chromatogram of urinary metabolites showing that hydrolysis of conjugated urinary metabolites of rats exposed to safrole resulted in 5 major metabolites including 1′-hydroxysafrole, 1,2-dihydroxy-4-allylbenzene and eugenol, which were identified based on a comparison to commercially available reference compounds, and two unidentified metabolites denoted M3 and M4.

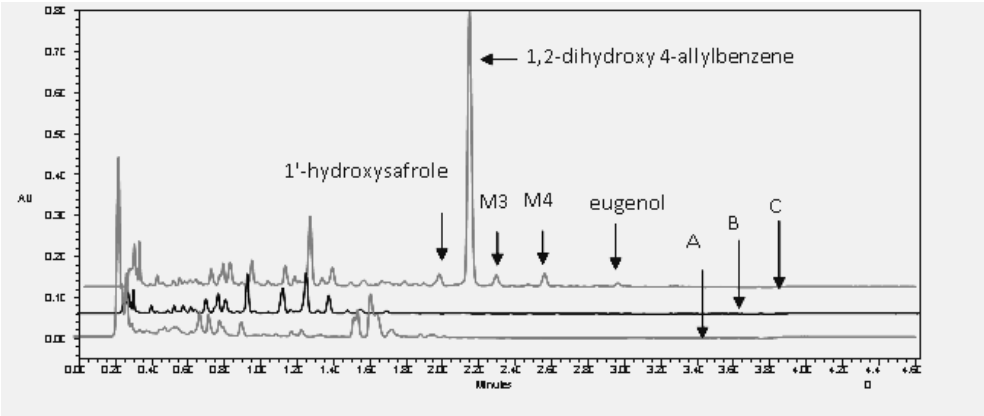

Figure 5.3: UPLC chromatogram of (A) unhydrolyzed urine, (B) hydrolyzed urine collected before gavage and (C) hydrolyzed urine collected 24-48 h after gavage from a rat exposed to 150 mg/kg bw safrole.

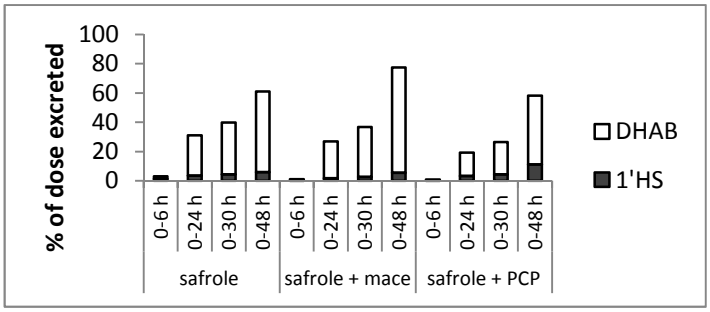

Figure 5.4. Cumulative (0-48 hours) excretion of the two major urinary safrole metabolites 1′-hydroxysafrole (1′HS) and 1,2-dihydroxy-4-allylbenzene (DHAB) following administration of 150 mg/kg bw safrole, 150 mg/kg bw safrole + 463 mg/kg bw mace extract, or 150 mg/kg bw safrole + 11 mg/kg bw PCP to rats.

Figure 5.4 shows the cumulative excretion (0-48 h after gavage) of the two major safrole metabolites 1′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene. The total percentage of the dose of safrole excreted in the 0-48 h urine as 1′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene amounted to 61.0±10.0%, 78.0±14.6% and 58.2±11.0% of the dose for rats dosed with 150 mg/kg bw safrole, 150 mg/kg bw safrole + 463 mg/kg bw mace extract or 150 mg/kg bw safrole + 11 mg/kg bw PCP, respectively. These data indicate that urinary

excretion of the major safrole metabolites is not significantly reduced when safrole is dosed in the presence of mace extract or PCP. This indicates that the reduction in DNA adduct formation is not due to a reduced bioavailability of safrole.

Refined risk assessment for safrole

The $BMDL_{10}$ values for safrole were calculated using BMD software version 2.4 based on the data from an in vivo study in female mice administered safrole via the diet for 3 days a week up to 52 weeks and for 86 weeks of study duration (Miller *et al.*, 1983). Table 5.1 presents an overview of the incidence of hepatocellular carcinomas in female mice with increasing doses of safrole as reported by Miller *et al.* (Miller *et al.*, 1983) and Table 5.2 presents the results of a BMD analysis of these data. The $BMDL_{10}$ values obtained amount to 2.6 to 7.0 mg/kg bw/day (Table 5.2). Based on the estimated dietary intake (EDI) for safrole reported by the SCF (2002) of 5 µg/kg bw/day, these $BMDL_{10}$ values result in MOE values that vary between 520 to 1400.

To estimate what $BMDL_{10}$ values and MOE values would have been obtained when safrole would have been tested in the bioassays not as a pure compound but in the presence of mace extract, the percentage inhibition of DNA adduct formation by mace extract defined in the present study was used to refine the tumor data and the resulting risk assessment. Table 5.1 presents the refined incidence of hepatocellular carcinomas calculated assuming that malabaricone C-containing mace extract would be co-administered to the mice together with safrole at a ratio of safrole to mace extract of 0.5 mg/µmol. These refined incidences of hepatocellular carcinomas at the respective dose levels of safrole were calculated using the reduction by 55 % in DNA adduct formation as observed in the rat study. It is noted that this approach assumes, as a first approximation, that a decrease in S-3′-N^2-dGuo safrole DNA adduct formation results in a corresponding reduction in hepatocellular carcinoma formation and also that the matrix effects would be similar in mice and rats. Table 5.3 presents the results of the BMD analysis of the refined tumor data. The BMD analysis of the newly generated refined data resulted in $BMDL_{10}$ values that varied from about 17.3 to 22.8 mg/kg bw/day. Using these newly estimated refined $BMDL_{10}$ values and the EDI for safrole of 5 µg/kg bw/day (SCF, 2002), the MOE for safrole would amount to about 3460

to 4560. Clearly, co-administration of safrole together with mace extract is thus predicted to increase the $BMDL_{10}$ and MOE levels substantially pointing at a lower priority for risk management, than when the MOE calculation would be based on the tumor data obtained with pure safrole.

Table 5.1. An overview of the incidence of hepatocellular carcinomas in female mice exposed to safrole as reported by Miller *et al*. (Miller *et al*., 1983) and the refined incidence of hepatocellular carcinomas calculated taking into account the inhibition of safrole bioactivation by malabaricone C-containing mace extract.

Experimental dose (mg/kg diet)	Duration of exposure/sacrifice (weeks)	Time-adjusted dose (mg/kg bw/day)[1]	No. of animals	Hepatocellular carcinomas incidence	Refined hepatocellular carcinomas incidence[2]
0	52/86	0	50	0	0
2500	52/86	80	47	34	15
5000	52/86	160	49	39	18

[1] Dose levels were adjusted for dosing regimen as described in Materials and methods.
[2] Incidences were refined assuming a reduction in hepatocellular carcinoma incidences of 55% in line with the observed reduction in safrole bioactivation.

Tabel 5.2 Results from a BMD analysis of the data on the incidence of hepatocellular carcinomas in mice exposed to safrole (Miller *et al.*, 1983) (Table 5.1) using BMD software version 2.4, a BMD of 10% extra risk and default settings.

Model	No. of parameters	Log Likelihood	p-value	Accepted	BMD_{10} (mg/kg bw/day)	$BMDL_{10}$ (mg/kg bw/day)
Null	1	-101.20				
Full	3	-57.51				
Gamma	1	-54.25	0.17	Yes	8.6	7.0
Logistic	2	-64.97	0.00	No	-	-
LogLogistic	1	-52.69	0.83	Yes	3.9	2.6
LogProbit	2	-52.51	1.00	Yes	0.3	ND*
Multistage	1	-54,26	0.17	Yes	8.6	7.0
Probit	2	-64.91	0.00	No	-	-
Weibull	1	-54.26	0.17	Yes	8.6	7.0
Quantal-linear	1	-54.26	0.17	Yes	8.6	7.0

* Not determined. Benchmark dose computation failed. Lower limit includes zero

Tabel 5.3. Results from a BMD analysis for the refined incidence of hepatocellular carcinomas in female mice (Table 5.1) using BMD software version 2.4, a BMD of 10% extra risk and default settings.

Model	No. of parameters	Log Likelihood	p-value	Accepted	BMD_{10} (mg/kg bw/day)	$BMDL_{10}$ (mg/kg bw/day)
Null	1	-78.03				
Full	3	-61.65				
Gamma	1	-62.71	0.34	Yes	30.0	22.8
Logistic	2	-67.78	0.00	No	-	-
LogLogistic	1	-62.26	0.54	Yes	24.6	17.3
LogProbit	2	-61.65	1.00	Yes	1.09	ND*
Multistage	1	-62.71	0.34	Yes	30.0	22.8
Probit	2	-67.19	0.00	No	-	-
Weibull	1	-62.71	0.34	Yes	30.0	22.8
Quantal-linear	1	-62.71	0.34	Yes	30.0	22.8

*Not determined. Benchmark dose computation failed. Lower limit includes zero.

Discussion

The results of the present paper show that malabaricone C-containing mace extract is able to inhibit formation of S-3′-N^2-dGuo safrole DNA adducts in the liver of SD rats exposed to safrole and mace extract simultaneously. To date only PCP, a well-known SULT inhibitor, demonstrated inhibition of the formation of safrole-DNA adducts in rodent liver (Boberg *et al.*, 1983; Daimon *et al.*, 1997; Randerath *et al.*, 1984). A great number of literature studies reported in vitro data on dietary compounds inhibiting SULTs such as epicatechin gallate (ECG), epigallocatechin gallate (EGCG), quercetin, kaempferol, genistein and daidzein (Coughtrie and Johnston, 2001; De Santi *et al.*, 2002; Mesia-Vela and Kauffman, 2003), various beverages containing flavonoids (Nishimuta *et al.*, 2007), basil extract (Alhusainy *al.*, 2010; Jeurissen *et al.*, 2008), and malabaricone C-containing mace extract (Martati *et al.*, 2012). The results of the present study demonstrate that the inhibition of safrole DNA adduct formation by malabaricone C-containing mace extract observed in in vitro model systems (Martati *et al.*, 2012) is also observed in vivo.

Using a previously developed PBBK model it was predicted that inhibition of the formation of 1′-sulfooxysafrole at a ratio of mace extract and safrole of 0.5 mg/μmol would amount to 80% under the assumption of 100% bioavailability of the mace extract constituents (Martati *et al.*, 2012). The present in vivo study demonstrates that the inhibition of S-3′-N^2-dGuo safrole DNA adduct formation in the liver of Sprague-Dawley rats by the mace extract amounted to 55%. The PBBK model predicted that a 55% inhibition of safrole bioactivation to 1′-sulfooxysafrole would be obtained when the bioavailability of the SULT inhibitors in the malabaricone C mace extract would be 68% instead of 100%, which may not be unrealistic. Thus, the results of the in vivo study are in line with what would be expected based on the PBBK model based predictions for safrole bioactivation.

It is important to note that the safrole DNA adduct measured in the present study is S-3′-N^2-dGuo which was shown to be the major adduct formed in vitro, in rodent studies and in oral cancer patients having a habit of betel quid chewing (Chung *et al.*, 2008; Daimon *et al.*, 1998; Phillips *et al.*, 1981; Randerath *et al.*, 1984). Given that all safrole DNA adducts are expected to be derived from the same 1′-sulfooxy metabolite it can be foreseen that the inhibition of the formation of this major S-3′-N^2-dGuo adduct also reflects inhibition of the formation of possible other minor safrole DNA adducts.

Previous in vitro studies already revealed that the inhibition of DNA adduct formation by malabaricone C-containing mace extract occurs at the level of the SULT mediated bioactivation of 1′-hydroxysafrole to 1′-sulfooxysafrole (Martati *et al.*, 2012). In the present study, it was demonstrated that urinary excretion of the major safrole metabolites, (conjugated) 1′-hydroxysafrole and 1,2-dihydroxy-4-allylbenzene is not significantly reduced when safrole is dosed in the presence of mace extract or PCP. This observation excludes that the reduction in DNA adduct formation is due to a reduced bioavailability of safrole when dosed together with malabaricone C-containing mace extract or PCP. The effect of PCP on safrole DNA adduct formation in the liver is in line with previous studies reporting a reduction in safrole DNA adduct formation upon co-administration of safrole with PCP, a known SULT inhibitor (Boberg *et al.*, 1983; Daimon *et al.*, 1997; Randerath *et al.*, 1984). Although the inhibition in the formation of S-3′-N^2-dGuo DNA adduct levels was ascribed to inhibition of the SULT mediated conversion of 1′-hydroxysafrole to 1′-

sulfooxysafrole, inhibition of other enzymes involved in the bioactivation or detoxification of safrole by mace extract constituents may also occur. In vitro tests reported by Kimura *et al.* (Kimura *et al.*, 2010) showed that mace extract resulted in a potent inhibition of P450 3A4 and P450 2C9 with IC_{50} values of 1.1-4.2 µg/mL, and that 17 compounds isolated from mace inhibited the activities of P450 3A4 and P450 2C9 with IC_{50} less than 11.2 µM. Since P450 2C9 was previously shown to be a major P450 enzyme involved in the 1′-hydroxylation safrole (Jeurissen *et al*., 2004; Ueng *et al*., 2004), this inhibition of P450 2C9 may have contributed to the observed reduction in safrole bioactivation by mace extract.

A question of interest is how to integrate the food matrix dependent modulation of safrole bioactivation in the risk assessment of safrole. This can be done using the so-called Margin of Exposure (MOE) approach and taking into account the results of the present study for the effect of the malabaricone C-containing mace extract on safrole bioactivation. Assuming a reduction in hepatocellular carcinoma incidences in line with the observed 55% reduction in safrole bioactivation, the incidences for hepatocellular carcinomas were refined resulting in $BMDL_{10}$ values of 17.3 to 22.8 mg/kg bw/day instead of 2.6 to 7.0 mg/kg bw/day and in MOE values that amount to 3460 to 4560 instead of 520 to 1400. From this analysis, it can be concluded that when safrole would have been tested in rodent bioassays in the presence of malabaricone C-containing mace extract, increased $BMDL_{10}$ and increased MOE values would have been obtained indicating a lower priority for risk management compared to the $BMDL_{10}$ and MOE values derived from rodent carcinogenicity data obtained when testing safrole as a pure compound.

In conclusion, the present study demonstrated that formation of safrole DNA adducts in the rat liver was reduced when safrole was administered in the presence of malabaricone C-containing mace extract. The results obtained point at a potential reduction of the cancer risk resulting in increased MOE values and a lower priority for risk management when the carcinogenicity of safrole would have been tested within a relevant food matrix containing SULT inhibitors compared to what is observed in rodent bioassays upon exposure to pure safrole dosed by gavage.

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CHAPTER 6

General discussion

General discussion

Safrole, 4-allyl-1,2-methylenedioxy-benzene, is a genotoxic carcinogen of which the use in food has been prohibited in the U.S. by FDA since 1960 and in Europe since 2008 (European Commission, 2008). As a result, main exposure results from dietary consumption of safrole containing herbs and spices, such as nutmeg, mace, star anise, pimento, cinnamon, and black pepper, and food products containing these herbs and spices or their essential oils. Herbs and spices or essential oils containing safrole are used in various foods, including for example meat products, baked goods, alcoholic beverages, soups and non-alcoholic beverages such as cola drinks (SCF, 2002; Siano *et al.*, 2003). At present exposure to safrole occurs primarily from (1) consumption of spices containing safrole especially mace or nutmeg, (2) consumption of cola beverages which may contain levels that are up to 3-5 times higher than the current EU regulation use limit of 1 mg/kg product (Choong and Lin, 2001), (3) consumption of alcoholic beverages since one out of sixteen tested commercial alcoholic beverages contained safrole higher than the current EU recommended limit of 5 mg/kg product (Curró *et al.*, 1987), (4) consumption of food products with added safrole containing spices such as Bologna sausages and Vienna sausage which have been reported to contain 0.2-2 mg safrole/kg product (Siano *et al.*, 2003), (5) consumption of plant food supplements containing safrole (Berg *et al.*, 2011) and (6) the habit of *Piper betle* chewing since betel quid may contain 15 mg/g fresh weight of safrole (Hwang *et al.*, 1992).

Various regulatory bodies have posed scientific opinions on the safety of safrole as food ingredient (SCF, 2002). The European Union′s Scientific Committee on Food (SCF) concluded that safrole has been demonstrated to be genotoxic and carcinogenic, and that therefore the existence of a threshold cannot be assumed so that the SCF could not establish a safe exposure limit. The SCF also indicated that consequently reductions in the exposure and restrictions in the use levels are indicated (SCF, 2002). The Committee of Experts on Flavouring Substances (CEFS) of the Council of Europe presented the same conclusion stating that efforts should be made to reduce the consumption of safrole via food and beverages as far as possible (Council of Europe, 1997). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded at its twenty-fifth meeting that

flavouring agents containing safrole as the principal flavour-active ingredient should not be used as food additives, and that it is not practicable to advocate the discontinuation of the use of spices containing safrole as minor constituents (e.g. nutmeg, mace and cinnamon) (JECFA, 1981). However, JECFA suggested that when these spices were used, the amount of safrole in the finished product should be kept as low as possible. The European Council Directive on food flavouring regulation (EC) no. 1334/2008/EEC that has been applied from January 20, 2011 onwards defined maximum levels of safrole, naturally present in flavourings that can be present in food ingredients with flavouring properties, as well as in certain compound food as consumed to which flavouring and/or food ingredients with flavouring properties have been added. The European Council defined a limit for safrole of 1 mg/kg for non-alcoholic beverages, 5 mg/kg for alcoholic beverages with more than 25% alcohol by volume and 15 mg/kg for meat preparation and meat products (European Commission, 2008). According to the FDA (webpage last updated in 2013)[1], the toxic effects of nutmeg and some of its active constituents such as myristicin, elemicin, and safrole are clearly manifested only at doses vastly greater than the realistic daily human intake. However, the essential oil of nutmeg contains 6% of safrole which is known as a weak hepatocarcinogen and a prohibited food additive in the U.S. since 1960. The intake level of nutmeg oil and its equivalent from nutmeg and mace, based on the total imports available annually for use in food in the U.S., is 0.05 mg per kg per day for adults (JECFA, 2009). Assuming a maximum content of 6 % safrole in the oil, the daily intake of safrole would be about 4 µg/kg bw/day for an adult. The Select Committee on GRAS Substances (SCOGS) concluded 1) that the safrole and myristicin content in nutmeg products, and perhaps the content of related constituents such as elemicin, eugenol, and methyleugenol, should be further investigated to determine the range of content of these constituents in both nutmeg and mace originating from the East and West Indian, 2) that it would be of use to investigate or reinvestigate their possible mutagenic, teratogenic, and carcinogenic effects, and 3) that there is no evidence that nutmeg, mace, or their essential oils

[1] http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/SCOGS/ucm260910.htm

demonstrate a hazard to the public as they are used in the manner now practiced. Uncertainties remain and therefore additional studies should be conducted (FDA CFR Section 182.10, 1973).

At present, there is no internationally harmonised approach for the risk assessment of compounds that are both genotoxic and carcinogenic. Several authorities use the Margin of Exposure (MOE) approach. The MOE is obtained by dividing the value of a $BMDL_{10}$ (the lower confidence bound of the benchmark dose that gives 10% extra cancer incidence (BMD_{10})) by the estimated daily intake (EDI) of the substance by the population of interest (EFSA, 2005; JECFA, 2005). This approach, however, does not result in a quantitative risk assessment at low dose levels representative for realistic daily human intake. At present, discussion is ongoing on how to translate the results from animal bioassays at high dose levels of the pure compound to the risk for the human population exposed to safrole at relatively low levels via dietary intake. This translation of cancer risks from the animal data to humans at relevant dietary intake levels needs a better understanding of species dependent, dose dependent, and interindividual dependent differences in bioactivation and detoxification of safrole and the possible influence of the food matrix on these processes. The aim of this thesis was to obtain insight into the dose-, species-, interindividual- and matrix dependent effects on the bioactivation and detoxification of safrole using physiologically based biokinetic (PBBK) modeling.

After an introduction to the thesis in chapter 1, in chapter 2, a PBBK model for detoxification and bioactivation of safrole in male rats was developed based on the model for the related alkenylbenzenes estragole and methyleugenol (Al-Subeihi *et al.*, 2011; Punt *et al.*, 2007). The PBBK model consists of eight compartments which are grouped into metabolizing compartments including liver, kidney, and lung, other compartments representing either separate tissues (including fat, venous and arterial blood), or tissues lumped together in compartments representing richly perfused and slowly perfused tissues. The model was built based on in vitro and in-silico derived parameters determined in the present thesis. Kinetics for relevant cytochrome P450 (P450) mediated conversions of safrole and conjugation of 1′-hydroxysafrole were determined in vitro using tissue fractions of organs that appeared capable of metabolizing safrole. The physiological parameter

values (e.g. organ volumes, cardiac output, and blood flows) were taken from literature (Brown *et al.*, 1997). Physico-chemical parameters (e.g. blood/tissue partition coefficients were obtained using in-silico models.

The performance of the model was evaluated by comparison of predicted levels of formation of 1,2-dihydroxy-4-allylbenzene (DHAB), 1′-hydroxysafrole glucuronide and total urinary safrole metabolites to the reported levels of these metabolites in urine of safrole exposed rats. This evaluation revealed that the predictions adequately matched with observed experimental values. The rat PBBK model predicted that at a low dose of 0.05 mg/kg bw and also at a high dose of 300 mg/kg bw, P450 mediated conversion of safrole mainly occurs in the liver with DHAB predicted to be the major P450 metabolite of safrole. A dose dependent shift in P450 mediated oxidation leading to a relative increase in bioactivation at high doses was not observed which is in contrast to outcomes previously obtained from similar PBBK models for the related alkenylbenzenes estragole and methyleugenol (Al-Subeihi *et al.*, 2011; Punt *et al.*, 2008). This can be explained by the fact that for P450 mediated conversion of safrole no saturation of an important metabolic route occurs in a dose range from 0.05 up to 300 mg/kg bw. Extrahepatic conversion of safrole did not contribute significantly to the overall metabolism. In male rat liver, glucuronidation is the major pathway for the conversion of 1′-hydroxysafrole followed by oxidation, whereas sulfation of 1′-hydroxysafrole to the ultimate carcinogenic metabolite 1′-sulfooxysafrole is a minor pathway.

In the risk assessment, when extrapolating from animal experimental data to the human situation, species differences in metabolic activation and detoxification of safrole should be taken into account. In chapter 3, the kinetic constants for bioactivation and detoxification of safrole with human tissue fractions were obtained. Furthermore, with the PBBK model for human defined in chapter 3, the formation of the proximate carcinogenic metabolite, 1′-hydroxysafrole, and of the ultimate carcinogenic metabolite, 1′-sulfooxysafrole, in human could be predicted.

The PBBK model for human was evaluated by comparison of the PBBK model based predicted and the reported experimental data on the level of total safrole metabolites

detected in the urine of humans exposed to dose levels of 0.0027 mg/kg bw (for a 60 kg person corresponding to 0.163 mg/subject) and 0.0276 mg/kg bw (for a 60 kg person corresponding to 1.655 mg/subject) (Benedetti *et al.*, 1977). This comparison revealed that the prediction adequately matched observed experimental values.

Results from the PBBK model based predictions for humans were compared to the PBBK model based predictions for rat to obtain insight in the possible interspecies differences in bioactivation of safrole. This comparison showed that the predicted level of formation of 1′-hydroxysafrole in human liver is fourfold higher than that for rat liver and that the predicted formation of 1′-sulfooxysafrole in human liver is about fivefold higher than that for rat liver. This indicates that the interspecies differences in toxicokinetics for bioactivation of safrole between rat an human are in line with the uncertainty factor normally taken into account for interspecies differences in toxicokinetics of 4 (IPCS, 2010). Species differences between human and rat in the nature of the detoxification pathways of 1′-hydroxysafrole were larger, with the formation of 1′-oxosafrole being the main detoxification pathway in humans but a minor pathway in rats and glucuronidation of 1′-hydroxysafrole being less important in humans than in rats.

At a dose relevant for human dietary intake of 0.005 mg/kg bw, formation of 1′-sulfooxysafrole in human liver was predicted to amount to 0.02 nmol/kg bw and this level increases to 8.7 nmol/kg bw at a dose level of 3 mg/kg bw. This shows that the formation of 1′-sulfooxysafrole in percentage of the dose at the low and high dose level of safrole was not significantly different. Plotting the dose of safrole (mg/kg bw) vs formation of 1′-sulfooxysafrole (µmol/kg bw) on a log-log scale for human and rat liver as shown in Fig. 6.1, shows a linear relationship over the dose range from dose levels known to cause cancer in rodent bioassays (for example 100 mg/kg bw) down to dose levels relevant for daily human intake and as low as the virtual safe dose (VSD). The VSD is a dose that is expected to give one additional cancer risk in a million people upon life time exposure (Edler *et al.*, 2002). By linear extrapolation from the BMD_{10} (Benchmark Dose causing 10% extra risk) obtained from a BMD analysis of the data from the in vivo study of Miller *et al*. (1983) and Boberg *et al*. (1983), amounting to 4.6-39 mg/kg bw/day, the VSD can be derived and amounted to 0.046-0.390 µg/kg bw/day (Martati *et al.*, 2011). This graph supports that

possible nonlinear kinetics and species differences in kinetics should not be used as an argument against using a linear low-dose cancer risk extrapolation from animal data to the low-dose human situation. This shows that PBBK models can evaluate the interspecies differences and provide a useful tool in risk assessment of food-borne chemicals when evaluating human risks.

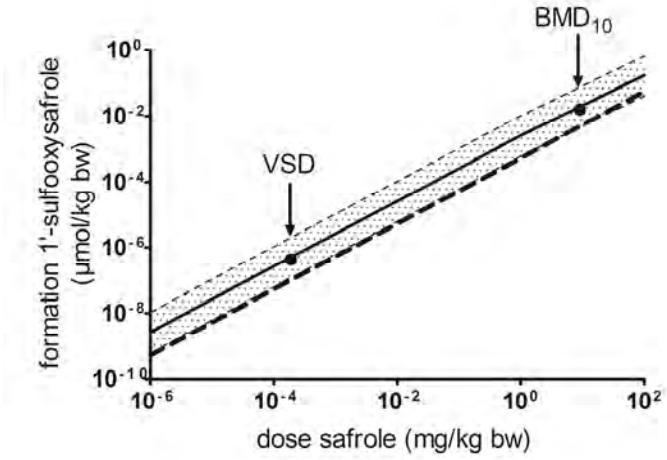

Figure 6.1. PBBK model-based predicted dose-dependent formation of 1′-sulfooxysafrole in the liver of human, corresponding to the median or 50th percentile (———), the 5th and 95th percentiles (– –) and rat (— —). Note that the lines of the 5th percentile of 1′-sulfooxysafrole formation in humans and the mean of those in rats overlap.

Another factor that should be taken into account in the risk assessment of safrole is the influence of human interindividual differences and this was studied in more detail also in chapter 3. Human variability may arise from a variety of sources including variation in physiological parameters or in the activity of metabolizing enzymes involved in safrole metabolism. To obtain insight in this human interindividual variability in metabolic activation and detoxification of safrole, Monte Carlo simulations were performed to assess the impact of the variability on the model predictions. For the Monte Carlo simulation, probability distributions were assigned to those parameters to which the model outputs for the predicted level of formation of the proximate carcinogenic metabolite, 1′-hydroxysafrole, and of the ultimate carcinogenic metabolite, 1′-sulfooxysafrole, were shown to be the most sensitive in the sensitivity analysis.

Monte Carlo simulations revealed that the formation of 1′-sulfooxysafrole was predicted to vary 4- to 17-fold in the population (fold-difference between the 95^{th} and median, and 95^{th} and 5^{th} percentile respectively). Moreover, a CSAF (chemical-specific adjustment factor) for interindividual differences in kinetics for predicted formation of 1′-sulfooxysafrole representing a ratio of the parameter estimate at the 95^{th} percentile and the median is 3.9 (IPCS, 2005). Further analysis reveals that the default uncertainty factor of 3.16 for interspecies variability in biokinetics (assuming that a factor of 10 is subdivided equally into two sub-factors each of $10^{-0.5}$ (3.16) for biokinetic and for biodynamic) (IPCS, 2010) adequately protects 91% of the population. It can also be concluded that for protecting 99% of the population a larger uncertainty factor for interindividual differences in biokinetics of 6.8 would be required.

It is of interest to note that the PBBK models for safrole developed in the present thesis describe the biokinetics of safrole. In a previous study on estragole, we extended the PBBK model to a so-called physiologically based biodynamic (PBBD) model describing the dose dependent DNA adduct formation. This was done by linking the PBBK model based predictions for formation of 1′-hydroxyestragole to DNA adduct formation in the liver by using data from an in vitro hepatocyte model in which the 1′-hydroxyestragole concentration dependent formation of DNA adducts was quantified (Paini *et al.*, 2010). This PBBD model for in vivo DNA adduct formation was validated using results from an in vivo study in which rats were exposed to estragole (Paini *et al.*, 2012). Performing similar in vitro studies to define an equation for the 1′-hydroxysafrole dependent formation of safrole DNA adducts in rat hepatocytes would allow extension of the current PBBK models predicting 1′-hydroxysafrole and 1′-sulfooxysafrole formation to a PBBD model predicting DNA adduct levels in the liver of safrole exposed rats.

A next step would be to correlate the number of DNA adducts to mutagenicity or even to the level of tumor formation. Such an attempt to relate the level of DNA adducts formed to tumor incidence in rats and mice exposed to carcinogenic compounds was reported before (Otteneder and Lutz, 1999). The results of this analysis revealed that the calculated adduct concentration responsible for a 50% hepatocellular tumor incidence appeared to vary with the carcinogen studied ranging from 53 to 2083 and 812 to 5543 adducts per 10^8 nt for rat

liver and mice liver, respectively. Likewise Paini *et al.* (Paini *et al.*, 2011) concluded that the number of DNA adducts estimated to be formed at the $BMDL_{10}$ for tumor formation of a series of liver carcinogens varied orders of magnitude. Based on that result, these authors concluded that the quantity of DNA adducts formed by a DNA-reactive compound is not a carcinogenicity predictor by itself but that other factors such as type of adduct and mutagenic potential of the adduct or other factors contributing to the mode of action for tumor formation may be equally relevant. Thus, characterization of the mutagenic and carcinogenic potential of the safrole DNA adducts formed is a topic that remains open for future studies.

The current EU Regulation No 1334/2008 prohibits addition of safrole as pure compound to foods in order to reduce its daily intake and increase the MOE values. As a result of these regulations, the current human intake of safrole mainly results from consumption of spices containing safrole such as mace, nutmeg, pepper, etc or from food to which those spices or their essential oils are added to replace the addition of pure safrole the use of which in food or beverages has been banned. Thus, human exposure to safrole is always within the context of a food matrix. In contrast to this, studies on the toxicity and carcinogenicity of safrole are generally performed using pure safrole. The presence of the complex food matrix may modulate the bioactivation and detoxification of safrole thereby influencing its toxicity. Therefore, risk assessment resulting from consumption of herbs and spices that contain safrole should preferably be performed taking into account the possible modulating effects of other compounds present in these herbs or spices. Previously, a scientific opinion on a guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements published by EFSA (EFSA, 2009), already indicated that where a matrix effect is advocated to support the safety of specific levels of substances (e.g. that data from a pure substance may overestimate effects of the substance in the botanical matrix), testing and/or other data should be provided to demonstrate the occurrence of the matrix effect of the preparation and its magnitude. EFSA also indicated that a matrix effect should be judged on a case-by-case basis (EFSA, 2009). The experiments described in chapter 4 of this thesis aimed at obtaining insight in possible matrix derived effects on the bioactivation of safrole. To this end, the inhibition of

sulfotransferase (SULT)-mediated bioactivation of 1′-hydroxysafrole by a methanolic mace extract was quantified. To this end the methanolic mace extract was tested for inhibition of SULT activity and subsequently fractionated testing also its fractions for SULT inhibition. In the mace fraction, the highest SULT inhibiting activity malabaricone C was identified as the relevant SULT inhibitor. The K_i (inhibition constant) for inhibition of SULT activity by the malabaricone C-containing mace extract was 8.3 and 1.0 mg/L for rat and human liver S9, respectively. At a methanolic mace extract concentration of 100 mg/mL, oxidation and glucuronidation of 1′-hydroxysafrole were not inhibited in incubations with human liver S9, showing that at these concentrations, the methanolic mace extract selectively inhibited only SULTs without modify the detoxification reactions of 1′-hydroxysafrole.

In further studies described in chapter 4, it was investigated whether the malabaricone C-containing mace extract would be able to inhibit the SULT mediated bioactivation and subsequent DNA adduct formation of safrole in an intact cell system, using human HepG2 cells. Exposure of HepG2 cells to 1′-hydroxysafrole in the presence of increasing concentrations of malabaricone C-containing mace extract revealed a dose-dependent inhibition of N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine (S-3′-N^2-dGuo) formation. With mace extract concentrations of 25 and 100 mg/L, S-3′-N^2-dGuo formation in HepG2 cells exposed to 1′-hydroxysafrole was inhibited by 60 and 88 %, respectively. The mace extract did not display cytotoxicity up to concentrations of at least 100 mg/L which was the highest concentration tested.

To investigate if possible effects on safrole bioactivation to 1′-sulfooxysafrole by malabaricone C-containing mace extract could also be expected in vivo, the SULT inhibition was integrated into the PBBK model using the *Ki* values obtained with rat or human S9. The PBBK models thus obtained predicted that at a dose of 50 mg/kg bw safrole and at a ratio of malabaricone C-containing mace extract to safrole similar to the level of these constituents in mace (44 mg/µmol), inhibition of 1′-sulfooxysafrole formation by malabaricone C-containing mace extract for rats and humans amounts to 90 and 100% when the bioavailability of the mace extract ingredients would be 100%. When the bioavailability of the malabaricone C-containing mace extract would be 1% inhibition of 1′-

sulfooxysafrole formation was predicted to amount to 61 and 91%, for rats and humans, respectively.

This result points at a potential reduction of the bioactivation and resulting cancer risk when safrole intake results from the food matrix containing malabaricone C or other SULT inhibitors present in the regular daily diet. A great number of literature studies reported some natural dietary compounds to also inhibit SULT activity including compounds such as epicatechin gallate (ECG), epigallocatechin gallate (EGCG) (Coughtrie and Johnston, 2001; De Santi *et al.*, 2002), kaempferol, quercetin, genistein and daidzein (Mesia-Vela and Kauffman, 2003), various beverages containing flavonoids (Nishimuta *et al.*, 2007) and basil extract shown to contain the flavonoid nevadensin that inhibits SULT activity with a *Ki* values of 4 nM (Alhusainy *et al.*, 2010; Jeurissen *et al.*, 2008).

In additional studies of the present thesis, it was demonstrated that the inhibition of safrole DNA adduct formation by malabaricone C-containing mace extract observed in in vitro model systems can actually be demonstrated in an in vivo experiment. Chapter 5 presents the results of an in vivo study in which Sprague-Dawley rats were orally exposed to mace extract and safrole and the level of safrole DNA adduct formation in the liver was quantified and compared to the level of safrole DNA adducts detected in the liver of rats exposed to safrole only. The ratio of mace extract and safrole (mg/µmol) used in this in vivo study was 0.5 instead of 44 mg/µmol, the latter being the ratio mimicking the ratio of these constituents in mace extract. This lower dose of mace extract was chosen to avoid possible toxicity of the mace extract. At a dose of safrole of 150 mg/kg bw and a ratio of mace extract and safrole of 0.5 mg/µmol, the dose of mace extract would amount to 463 mg/kg bw. This is lower than the dose of 3000 mg/kg bw reported by El Malti *et al.* (2008) to result in death of mice orally exposed to mace extract, whereas at the lower dose of 300 mg/kg bw, the mice survived (El Malti *et al.*, 2008). At a dose safrole of 150 mg/kg bw, and a ratio of mace extract and safrole of 0.5 mg/µmol the predicted inhibition of 1′-sulfooxysafrole was still 80% compared to 90% for the ratio of 44 mg/µmol. Based on these considerations, the ratio of 0.5 mg mace extract/µmol was chosen for the in vivo study reported in chapter 5 of this thesis. The results of the in vivo study demonstrate that the inhibition of S-3′-N^2-dGuo safrole DNA adduct formation in the liver of Sprague-

Dawley rats by the mace extract amounted to 55%. The PBBK model predicted that a 55% inhibition of safrole bioactivation to 1′-sulfooxysafrole would be obtained when the bioavailability of the SULT inhibitors in the malabaricone C-containing mace extract would be 68% instead of 100%, which may not be unrealistic. Thus, the results of the in vivo study are in line with what would be expected based on the PBBK model based predictions for safrole bioactivation.

The in vivo study also revealed a 64% inhibition of the safrole DNA adduct formation in the liver upon co-exposure of the rats to safrole and the well-known SULT inhibitor PCP. This result is in line with results reported before in rats or mice exposed to safrole or 1′-hydroxysafrole in the presence of PCP (Boberg *et al.*, 1983; Daimon *et al.*, 1997; Randerath *et al.*, 1984).

In chapter 5, it was also demonstrated that urinary excretion of the major safrole metabolites, (conjugated) 1′-hydroxysafrole and DHAB was not significantly reduced when safrole was dosed in the presence of mace extract or PCP. This observation excludes that the reduction in DNA adduct formation is due to a reduced bioavailability of safrole when dosed together with malabaricone C-containing mace extract or PCP.

Inhibition of other enzymes involved in the bioactivation or detoxification of safrole by mace extract constituents may also occur. In vitro tests reported by Kimura *et al*. (2010) showed that mace extract resulted in a potent inhibition of P450 3A4 and P450 2C9 with IC_{50} values of 1.1-4.2 µg/mL, and that 17 compounds isolated from mace inhibited the activities of P450 3A4 and P450 2C9 with IC_{50} less than 11.2 µM (Kimura *et al.*, 2010). Since P450 2C9 was previously shown to be a major P450 enzyme involved in the 1′-hydroxylation safrole (Jeurissen *et al*., 2004; Ueng *et al*., 2004), this inhibition of P450 2C9 may have contributed to the observed reduction in safrole bioactivation by methanolic mace extract.

It is also of interest to note that the levels of safrole DNA adducts detected in the in vivo study upon a dose of 150 mg/kg bw safrole amounted to 2400$\pm$1200 adducts/10^8nt. This level is in line with the level of 3700-8000 adducts/10^8 nt previously detected for rats exposed to 150 mg/kg bw estragole (Alhusainy *et al.*, 2013; Paini *et al.*, 2012). It is also of

importance to note that these values are 2 orders of magnitude higher than the levels recently reported by Herrmann *et al*. (2013) for the DNA adduct levels of the related alkenylbenzene methyleugenol in liver samples of 30 human subjects amounting to values of 13 to 37 per 10^8 nt (for respective median and maximum values). The methyleugenol adducts detected in this human study are likely to result from intake of food containing methyleugenol. This comparison reveals that the DNA adduct levels in the liver of rats exposed to alkenylbenzenes at dose levels that are capable of inducing liver tumors are only two orders of magnitude higher than the levels apparently present due to current levels of dietary human exposure, suggestion in another way that the MOE values for these compounds that are genotoxic and carcinogenic may be (too) low and of safety concern.

Another issue that might be raised is whether results from studies testing safrole or the other alkenylbenzenes as pure compounds, without the normal food matrix being present, provide an adequate starting point for risk assessment. Instead one may develop ways to integrate the food matrix dependent modulation of safrole bioactivation in the risk assessment of safrole. This can be done using the so-called Margin of Exposure (MOE) approach and taking into account the results of the in vivo study for the effect of the malabaricone C-containing mace extract on safrole bioactivation presented in chapter 5 of this thesis. Assuming a reduction in hepatocellular carcinoma incidences in line with the observed 55% reduction in safrole bioactivation, the incidences for hepatocellular carcinomas were refined resulting in $BMDL_{10}$ values of 17.3 to 22.8 mg/kg bw/day instead of 2.6 to 7.0 mg/kg bw/day and in MOE values that amount to 3460 to 4560 instead of 520 to 1400. From this analysis, it can be concluded that when safrole would have been tested in rodent bioassays in the presence of malabaricone C-containing mace extract increased $BMDL_{10}$ and increased MOE values would have been obtained indicating a lower priority for risk management compared to the $BMDL_{10}$ and MOE values derived from rodent carcinogenicity data obtained when testing safrole as a pure compound.

It is important to note that the inhibition of safrole DNA adduct formation by malabaricone C-containing mace extract in the present thesis was shown at high dose levels of safrole and malabaricone C-containing mace extract. The PBBK models developed in the present thesis may also be applied to investigate to what extent the inhibition of safrole bioactivation by

malabaricone C-containing mace extract would be observed at lower realistic levels of dietary intake. It could be calculated that at a dose level of 0.05 mg/kg bw safrole and 13.6 mg/kg bw malabaricone C-containing mace extract, reflecting their ratio in mace of 44 mg/µmol, the inhibition of 1′-sulfooxysafrole formation would amount to 23% in rat and 77% in human liver. However, at realistic human dietary intakes the food matrix is likely to contain also a variety of other SULT inhibitors including epicatechin gallate (ECG), epigallocatechin gallate (EGCG) (Coughtrie and Johnston, 2001; De Santi *et al.*, 2002), kaempferol, quercetin, genistein and daidzein (Mesia-Vela and Kauffman, 2003), various beverages containing flavonoids (Nishimuta *et al.*, 2007) and basil extract shown to contain the flavonoid nevadensin that inhibits SULT activity with a *Ki* values of 4 nM (Alhusainy *et al.*, 2010; Jeurissen *et al.*, 2008). It may be expected that the combined effect of all these SULT inhibitors may result in an inhibition of SULT mediated safrole bioactivation also at dose scenarios reflecting realistic human intake via food. This remains to be investigated.

Future perspectives and conclusions

The present thesis describes the predictions of the dose-, species, interindividual-, and matrix- dependent effects on the bioactivation and detoxification of safrole using PBBK modeling. Several issues remain to be developed to a further extent to completely understand the factors affecting the ultimate risk assessment of dietary human exposure to safrole. In this section some factors to be considered in future research and risk assessment of safrole are discussed in some more detail.

1. Improving the PBBK model by incorporation of a sub-model for malabaricone C and of possible P450 inhibition.

The present PBBK-based prediction for the inhibition of in vivo safrole DNA adduct formation by malabaricone C-containing mace extract matched relatively well with the values actually observed in an in vivo validation experiment. However, further optimization of the model, incorporating a sub-model for malabaricone C kinetics may further improve the predictions. This was recently shown for the inhibition of estragole DNA adduct formation by the basil ingredient nevadensin. Incorporation of a nevadenisin PBBK sub-model into the estragole model significantly improved the predictions resulting in a less

than 2-fold difference between predicted and actually observed DNA adduct levels (Alhusainy *et al.*, 2013). Such studies will require purification of malabaricone C and testing of the pure compound which also could be an important objective for further studies.

A further refinement of the PBBK model could be to include inhibition of P450 by mace extract or the newly identified SULT inhibitor since such effects may also influence the bioactivation of safrole. Kimura *et al.* (Kimura *et al.*, 2010) showed that extracts from 25 herbs and spices contained inhibitors of P450 3A4 and P450 2C9 and that mace extract was one of them resulting in approximately 95% inhibition at concentrations of mace extract of 100 µg/mL for the P450s tested. Mace extract selectively inhibited P450 3A4 and P450 2C9 with IC_{50} values of 3.9 and 1.1 µg/mL, respectively. Moreover, the seventeen isolated compounds from this mace extract were shown to have IC_{50} values for inhibition of P450 3A4 and P450 2C9 ranging from 0.24 to >100 µM. Based on these results, it can be foreseen that in the presence of mace extract the inhibition of P450 mediated conversion of safrole may occur, which may affect the ultimate metabolite patterns. Once established the kinetics for inhibition of P450 activity by malabaricone C-containing mace extract and/or pure malabaricone C can be integrated into the PBBK model, to further improve its predictive power.

2. Development of a physiologically based biodynamic (PBBD) model for safrole

The PBBK models obtained for safrole predict the kinetic characteristics of bioactivation and detoxification of safrole allowing evaluation of dose-, species-, interindividual- and even matrix- dependent effects on the bioactivation and detoxification of the compound. The ultimate carcinogenic effect of safrole will depend not only on these kinetic characteristics but also on toxicodynamic processes, such as the formation of DNA adducts, as well as the nature, repair and mutagenicity of the adducts formed and the processes ultimately leading to cancer formation. Therefore, information related to the formation of DNA adducts, the biological processes of injury, DNA repair, and tumor formation, and development should be considered. This can be done by extending the present PBBK models to physiologically based bioynamic (PBBD) models for safrole. In a first step, the PBBK model can be extended to a PBBD model by including an equation describing the

relationship between formation of 1′-hydroxysafrole or 1′-sulfooxysafrole and DNA adduct formation. An example of such a PBBD model can be found in the PBBD model developed for estragole describing the dose-dependent formation of DNA adducts of estragole using the PBBK model for estragole and coupling it to in vitro data obtained with rat primary hepatocytes (Paini *et al.*, 2010). The predicted formation of estragole DNA adducts in the liver of rats was shown to match the in vivo results. Such a PBBD model for safrole is obviously needed to proceed at least one step beyond the bioactivation to 1′-sulfooxysafrole and predict DNA adduct formation. As stated already above characterization of the mutagenic and carcinogenic potential of the safrole DNA adducts formed is a topic that remains open for future studies.

3. Identification of the SULT enzymes for safrole bioactivation and their inhibition by malabaricone C

Based on the results of the present study the inhibition of the formation of safrole DNA adducts by a methanolic mace extract was attributed to the inhibition of the SULT mediated conversion of 1′-hydroxysafrole to 1′-sulfooxysafrole. Co-administration of safrole and the malabaricone C-containing mace extract or the SULT inhibitor PCP to rats (present thesis) and of safrole and PCP to mice (Boberg *et al.*, 1983) resulted in the reduction of DNA adduct formation in the liver as a result of inhibition of SULT mediated formation of 1′-sulfooxysafrole. From the sensitivity analysis of the PBBK model for rats and humans, it could be derived that formation of 1′-sulfooxysafrole will be most affected by the kinetic parameters of sulfation of 1′-hydroxysafrole. Human has at least 11 different SULTs enzymes and it would be of interest to identify the SULT enzymes involved in safrole bioactivation because that would allow to take consequences of possible genetic polymorphisms in relevant SULTs into account in the risk assessment. Identification of the SULTs able to activate 1′-hydroxysafrole to 1′-sulfooxysafrole could be performed as done previously for methyleugenol using *Salmonella typhimurium* strains expressing various human or rodent SULTs developed by Herrmann *et al*. (2012). Using these modified *Salmonella typhimurium* strains for Ames tests on 1′-hydroxymethyleugenol and its isomers, human SULT1A1 and SULT1C2 were observed to be the most active SULTs in the formation of DNA reactive methyleugenol metabolites (Herrmann *et al.*, 2013). Using

these modified *Salmonella typhimurium* strains for Ames tests on 1′-hydroxysafrole will provide information of the SULTs involved in the bioactivation and mutagenicity of safrole. Using 1′-hydroxysafrole in incubations with SULT1A1 Chung *et al*. (2009) showed that 1′-hydroxysafrole can be efficiently sulfonated by SULT1A1 (Chung *et al.*, 2009). Identification of the actual SULT enzyme involved and of its possible inhibition by malabaricone C remains of interest for further studies.

4. Risk assessment of the related alkenylbenzenes using read-across of the PBBK models

It is of interest to compare the results of the PBBK model for safrole to those obtained with the previously developed PBBK model for the related alkenylbenzenes estragole and methyleugenol in rats and humans (Punt *et al*., 2008; Punt *et al*., 2009; Al-Subeihi *et al*., 2011; Al-Subeihi *et al*., 2012). This will reveal insight in their relative bioactivation and chances on DNA adduct formation and can be compared to possible differences in their $BMDL_{10}$ values for tumor formation. The comparison of the PBBK model based predicted formation of the ultimate carcinogenic 1′-sulfooxy metabolites of safrole, methyleugenol and estragole appeared to vary less than 6-fold in male rat liver and to vary even less by 2- to 5-fold in human liver. Formation of DNA adducts in HepG2 cells exposed to the alkenylbenzenes varied 3.5-fold (Zhou *et al.*, 2007). Furthermore, the calculated $BMDL_{10}$ values showed a comparable carcinogenic potency amounting to 3-29 (Martati *et al.*, 2011) mg/kg bw/day, 22-32 mg/kg bw (Rietjens *et al.*, 2008), and 9-33 mg/kg bw/day (Rietjens *et al.*, 2010) for safrole, methyleugenol, and estragole, respectively. Comparison of the predicted level of formation of the ultimate carcinogenic 1′-sulfooxy metabolites obtained from the developed PBBK models to the other in vitro or in vivo data available indicated that safrole, methyleugenol and estragole have a comparable carcinogenic potential. Taking this observation into account it is concluded that based on PBBK model based prediction of detoxification and bioactivation of related alkenylbenzenes one could apply read across and predict the carcinogenic potential for alkenylbenzenes for which no rodents tumor data are available, like for example elemicin and apiole, without the need for further animal testing. Thus PBBK model based predictions could facilitate risk assessment without the need for animal experiments. Recently, van den Berg *et al*. (2012) provided an example of this approach and performed a risk assessment of the alkenylbenzene elemicin for which in vivo

rodent carcinogenicity data are not available using PBBK model based read-across to the structurally related estragole and methyl eugenol for which tumor data are reported.

5. Formation of safrole derived quinone metabolites by further conversion of DHAB and their mutagenic potency

Another issue that might be considered in future work on safrole but also on the read across to other alkenylbenzenes would be the role of the *ortho*-quinone and/or *para*-quinone methide metabolites that have been reported to be formed from the safrole metabolite DHAB. These reactive quinone/quinone methide electrophiles may be scavenged via a reaction with glutathione (GSH) but may also cause further toxicity through either their potential to support redoxcycling and oxidative stress or to form covalent adducts with cellular macromolecules (Bolton *et al.*, 1994; O'Brien, 1991). Lee-Chen *et al*. (Lee-Chen *et al.*, 1996) reported that DHAB induced formation of the oxidative damage DNA product 8-hydroxy-2′-deoxyguanosine (8-OH-dG) which may be responsible for the increased mutagenicity and cytotoxicity in CHO-K1 cells and in the *S.typhimurium* TA102 test. Sugumaran and Bolton (1995) also reported production of *p*-quinone methide from DHAB exhibiting DNA binding potency. In rats exposed to safrole at dose levels of 500 and 1000 mg/kg bw/day for 5 days, 8-OH-dG levels increased and rapidly returned to a basal level on day 15 (Liu *et al.*, 1999). This is in contrast to the safrole DNA adducts formed in mice exposed to safrole that can still be detected in the liver at 30 and 140 days after dosing of 10 mg safrole/mouse (Gupta *et al.*, 1993; Randerath *et al.*, 1984). Based on theses observations one may suggest that the stable safrole DNA adducts may play a more important role in the initiation of cell lesions than the rapidly repaired safrole-induced 8-OH-dG although this remains to be investigated in more detail. The role of the DHAB derived quinone/quinone methide metabolites in the adverse effects of safrole may also be limited to some extent because DHAB can be excreted following its conjugation prior to oxidation or upon GSH conjugation of its quinone/ quinone methide which has been observed to occur in rat hepatocytes (Nakagawa *et al.*, 2009).

In conclusion, in the present thesis it has been shown that integrating in vitro metabolic, physiological, and biochemical parameters in PBBK models forms a good tool to give

insight into the possible dose-, species-, interindividual- and matrix dependent effects on the bioactivation of safrole. Furthermore, given the role of the SULT-mediated formation of safrole DNA adduct formation in the hepatocarcinogenicity of safrole in rodents, the results of the present study indicate that the likelihood of bioactivation and subsequent adverse effects in rodent bioassays will be lower when safrole is tested in the presence of a matrix containing SULT inhibitors such as malabaricone C-containing mace extract, compared to exposure of safrole as a pure compound. This will affect the risk assessment of safrole since it will result in increased $BMDL_{10}$ and MOE values pointing at a lower priority for risk management actions.

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CHAPTER 7

Summary

Summary

Safrole is a genotoxic carcinogen the use of which in human food has already been prohibited since 1960 in U.S. and since 2008 in Europe (FDA Ban 21 CFR 189, 180; revised April 1 2008; European Commission, 2008). As a result, the main exposure to safrole occurs through the use of herbs and spices containing low levels of safrole, such as nutmeg, mace, star anise, pimento, cinnamon, and black pepper, and food products containing these herbs and spices or their essential oils. Some foods and beverages contain safrole at levels higher than the current European Union (EU) recommended limit (Siano *et al*., 2003; Choong and Lin, 2001).

The safety of human exposure to safrole at low dietary intake levels has been assessed several times (Council of Europe, 1997; SCF, 2002; SCOGS, 1973). The Scientific Committee on Food (SCF) of the EU concluded in their evaluation that safrole is genotoxic and carcinogenic and that reductions in the exposure and restriction in the use levels are indicated (SCF, 2002). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded at its twenty-fifth meeting that flavouring agents containing safrole as the principal flavour-active ingredient should not be used as food additives (JECFA, 1981). These opinions are all based on carcinogenicity data from rodent studies as adequate human data were not available. Therefore, translation from animal bioassays at high dose levels of the pure compound to the risk for the human population exposed to safrole at relatively low levels via dietary intake within the complex food matrix is obviously needed. This translation needs a better understanding of species-, dose-, and interindividual dependent differences in bioactivation and detoxification of safrole and the possible influence of the food matrix on these processes. The aim of this thesis was to obtain insight into the dose-, species-, interindividual- and matrix dependent effects on the bioactivation and detoxification of safrole using physiologically based biokinetic (PBBK) modeling.

In the first chapter, background information of the topic and the aims of the thesis are presented. In chapter 2, a PBBK model for detoxification and bioactivation of safrole in male rats was developed based on in vitro metabolic parameters determined, in silico derived partition coefficients, and physiological parameter values taken from literature

(Brown *et al.*, 1997). The PBBK model consists of eight compartments including liver, kidney and lung as metabolizing compartments and compartments for fat, venous and arterial blood, richly perfused tissue and slowly perfused tissue. The performance of the model was evaluated by comparison of predicted levels of 1,2-dihydroxy-4-allylbenzene (DHAB), 1′-hydroxysafrole glucuronide (1′-HSG) and total urinary safrole metabolites to the reported levels of these metabolites in urine of rats exposed to safrole (Benedetti *et al.*, 1977; Klungsøyr and Scheline, 1983). This evaluation revealed that the predictions adequately matched observed experimental values. With the model obtained the relative extent of bioactivation and detoxification of safrole at different oral doses was examined. At low as well as high doses, cytochrome P450 (P450) mediated conversion of safrole mainly occurs in the liver in which DHAB was predicted to be the major P450 metabolite of safrole. A dose dependent shift in P450 mediated conversion leading to a relative increase in bioactivation at high doses was not observed. This can be explained by the fact that for P450 mediated conversion of safrole no saturation of an important metabolic route occurs in a dose range from 0.05 up to 300 mg/kg bw. Extrahepatic conversion of safrole did not contribute significantly to the overall metabolism. In male rat liver, glucuronidation is the major pathway for the conversion of 1′-hydroxysafrole followed by oxidation, whereas sulfation of 1′-hydroxysafrole to generate the ultimate carcinogenic metabolite 1′-sulfooxysafrole is a minor pathway.

Chapter 3 of the present thesis describes a PBBK model for safrole in human based on in vitro and in silico derived parameters. The model is composed of eight compartments including liver, kidney and lung as metabolizing compartments and compartments for fat, venous and arterial blood, richly perfused tissue and slowly perfused tissue. With the model obtained, the time- and dose-dependent formation of the proximate and ultimate carcinogenic metabolites, 1′-hydroxysafrole and 1′-sulfooxysafrole in human liver were estimated. In addition, a Monte Carlo simulation was performed to predict interindividual variation in the formation of these metabolites in the population as a whole. The PBBK model for human was evaluated by comparison of the PBBK predicted and the reported experimental data on the level of total safrole metabolites detected in the urine of human volunteers exposed to safrole (Benedetti *et al.*, 1977) which showed an adequately match.

The comparison of the PBBK model for rat and human revealed that the predicted level of formation of 1′-hydroxysafrole in human liver is fourfold higher than that for rat liver and the predicted formation of 1′-sulfooxysafrole is about fivefold higher than that for rat liver. This indicates that the interspecies differences in toxicokinetics for bioactivation of safrole between rat an human are in line with the uncertainty factor normally taken into account for interspecies differences in toxicokinetics of 4 (IPCS, 2010). Species differences between human and rat in the nature of the detoxification pathways of 1′-hydroxysafrole were larger, with the formation of 1′-oxosafrole being the main detoxification pathway in humans but a minor pathway in rats and glucuronidation of 1′-hydroxysafrole being less important in humans than in rats. The PBBK model predicted that the formation of 1′-sulfooxysafrole in the liver of human and rat shows a linear relationship over the dose range from dose levels known to cause cancer in rodent bioassays (100 mg/kg bw) down to dose levels relevant for daily human intake and as low as the virtual safe dose (VSD). This shows that PBBK models can evaluate the interspecies differences and provide a useful tool in risk assessment of food-borne chemicals when evaluating human risks. Monte Carlo simulations revealed that the formation of 1′-sulfooxysafrole was predicted to vary 4- to 17-fold in the population (fold-difference between the 95^{th} and median, and 95^{th} and 5^{th} percentile, respectively). Moreover, a CSAF (chemical-specific adjustment factor) for interindividual differences in kinetics for predicted formation of 1′-sulfooxysafrole representing a ratio of the parameter estimate at the 95^{th} percentile and the median is 3.9 (IPCS, 2005). Comparison of the CSAF with the default uncertainty factor of 3.16 for interspecies variability in biokinetics (assuming that a factor of 10 is subdivided equally into two sub-factors each of $10^{-0.5}$ (3.16) for biokinetic and for biodynamic) (IPCS, 2010) reveals that the default uncertainty factor adequately protects 91% of the population.

The current EU regulation No. 1334/2008 banned addition of safrole as pure compound to food in order to reduce its daily intake. As a result of these regulations, the current human intake of safrole mainly results from consumption of spices containing safrole such as mace, nutmeg, pepper, etc. or from food to which those spices or their essential oil are added. Therefore, risk assessment of safrole resulting from consumption of herbs and spices containing safrole should be performed taking into account the possible modulating effect

of other compounds present in these herbs or spices. The consequences of possible matrix dependent effects on the bioactivation and detoxification of safrole was investigated in chapter 4. Since mace is known to contain relatively high levels of safrole (Archer, 1988), methanolic mace extract and its fractionated samples were tested for inhibition of SULT activity, thereby inhibiting the final step in the bioactivation of safrole. The mace fraction containing malabaricone C showed the highest SULT inhibiting activity. The K_i (inhibition constant) for inhibition of SULT activity by the malabaricone C-containing extract was 8.3 and 1.0 mg/L for rat and human liver S9, respectively. Inhibition by mace extract of oxidation and glucuronidation of 1′-hydroxysafrole was not observed at a mace extract concentration of 100 mg/L showing that mace extract inhibited selectively SULTs. In chapter 4, further studies using human HepG2 cells exposed to 1′-hydroxysafrole and in the presence of mace extract showed that formation of the DNA adduct N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine (S-3′-N^2-dGuo) was inhibited by 60 and 80% at mace extract concentrations of 25 and 100 mg/L, respectively. The mace extract did not display cytotoxicity up to concentrations of at least 100 mg/L which was the highest concentration tested.

To investigate if possible effects on safrole bioactivation to 1′-sulfooxysafrole by malabaricone C-containing mace extract could also be expected in vivo, the SULT inhibition was integrated into the PBBK model using the K_i values obtained with rat or human S9. The PBBK models thus obtained predicted that at a dose of 50 mg/kg bw safrole and a ratio of malabaricone C-containing mace extract to safrole similar to the level of these constituents in mace (44 mg/μmol), inhibition of 1′-sulfooxysafrole formation by malabaricone C-containing mace extract for rats and humans amounts to 90 and 100%, respectively, when bioavailibility of the mace extract ingredients would be 100%. When bioavailibility of the mace extract ingredients would be 1%, the inhibition of 1′-sulfooxysafrole formation would be 61 and 91%, respectively. This indicates a potential reduction of the bioactivation and of the cancer risk when safrole intake results from the food matrix containing malabaricone C or other SULT inhibitors presents in the regular daily diet as compared to results obtained when dosing safrole as a pure compound.

In chapter 5, it was demonstrated that the inhibition of safrole DNA adduct formation by malabaricone C-containing mace extract observed in the in vitro model systems is also observed in an in vivo experiment. To this end Sprague-Dawley rats were orally exposed to mace extract and safrole and the level of safrole DNA adducts formed in the liver was quantified and compared to the level of safrole DNA adducts detected in the liver of rats exposed to safrole only. The results obtained demonstrate that safrole DNA adduct formation in the liver of Sprague-Dawley rats by the mace extract was reduced by 55%. The PBBK model predicted that a 55% inhibition of safrole bioactivation to 1′-sulfooxysafrole would be obtained when the bioavailability of the SULT inhibitors in the malabaricone C-containing mace extract would be 68% instead of 100%, which may not be unrealistic. Thus, the results of the in vivo study are in line with what would be expected based on the PBBK model based predictions for safrole bioactivation. The in vivo study also revealed a 64% inhibition of the safrole DNA adduct formation in the liver upon co-exposure of the rats with the well-known SULT inhibitor PCP. This result is in line with results reported before in rats or mice exposed to safrole or 1′-hydroxysafrole in the presence of PCP (Boberg *et al.*, 1983; Daimon *et al.*, 1997; Randerath *et al.*, 1984). It was also demonstrated in chapter 5 that urinary excretion of the major safrole metabolites, (conjugated) 1′-hydroxysafrole and DHAB was not significantly reduced when safrole was dosed in the presence of mace extract or PCP. This observation excludes that the reduction in DNA adduct formation is due to a reduced bioavailability of safrole when dosed together with malabaricone C-containing mace extract or PCP.

Chapter 6 of the thesis presents a general discussion of the results obtained. With the PBBK models developed for rats and humans, the time- and dose-dependent formation of the proximate and ultimate carcinogenic metabolites, 1′-hydroxysafrole and 1′-sulfooxysafrole in rat and human liver can be predicted. Human interindividual differences in bioactivation of safrole can be simulated using Monte Carlo simulation. Moreover, the predictions by the PBBK models for rat and human were compared to evaluate the occurrence of species differences in bioactivation and detoxification of safrole. The results of the in vitro and in vivo studies that demonstrated inhibition of the formation of safrole DNA adducts by mace extract, support that combination effects should be taken into account in the risk assessment

when safrole is tested in the presence of a relevant food matrix. To integrate the food matrix dependent modulation of safrole bioactivation in the risk assessment of safrole, the so-called Margin of Exposure (MOE) approach can be used. This revealed that when safrole would be tested in rodent bioassays in the presence of a matrix containing SULT inhibitors the MOE values would be higher and the need for risk management actions would be lower. Finally, chapter 6 presents also some issues of interest for further research on bioactivation and risk assessment of safrole.

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CHAPTER 8

Samenvatting

Samenvatting

Safrol is een genotoxisch carcinogeen waarvan het gebruik in voedingsmiddelen in de V.S. al sinds 1960 is verboden en sinds 2008 ook in Europa (FDA Ban 21 CFR 189, 180; revised April 1 2008; European Commission, 2008). Als gevolg daarvan vindt blootstelling aan safrol hoofdzakelijk plaats door het gebruik van kruiden en specerijen die lage doses safrol bevatten, zoals nootmuskaat, foelie, steranijs, piment, kaneel en zwarte peper en voedingsproducten die deze kruiden en specerijen of de essentiële oliën daarvan bevatten. Sommige voedingsmiddelen en dranken bevatten hogere niveaus safrol dan de huidige limiet die door de Europese Unie (EU) is aanbevolen (Siano *et al*., 2003; Choong and Lin, 2001).

De veiligheid van menselijke blootstelling aan safrol bij lage innameniveaus is meerdere keren onderzocht (Europese Commissie, 1997; SCF, 2002; SCOGS, 1973). De Scientific Committee on Food (SCF) van de EU stelde in diens evaluatie vast, dat safrol genotoxisch en carcinogeen is en dat reductie van de blootstelling en beperking in de gebruiksniveaus noodzakelijk is (SCF, 2002). De Joint FAO/WHO Expert Committee on Food Additives (JECFA) concludeerde in zijn 25^{e} bijeenkomst dat smaaktoevoegingen die hoofdzakelijk safrol als smaakbepalend ingrediënt bevatten, niet meer als voedseladditieven gebruikt zouden moeten worden (JEFCA, 1981). Deze opvattingen zijn allemaal gebaseerd op carcinogeniteitsgegevens verkregen uit knaagdierstudies omdat adequate menselijke gegevens niet beschikbaar waren. Daarom is er behoefte aan een vertaling van uitkomsten van dierlijke bioassays, uitgevoerd bij hoge doses van de pure stof, naar het risico voor de mens die wordt blootgesteld aan safrol bij relatief lage doses via inname in een complexe voedselmatrix. Voor deze vertaling is een beter begrip nodig van de van de species-, dosis- en individu-afhankelijke verschillen in bioactivering en detoxificering van safrol en de mogelijke invloed van de voedselmatrix op deze processen. Het doel van dit proefschrift is om inzicht te verkrijgen in dergelijke species-, dosis- en individu-afhankelijke verschillen in bioactivering en detoxificering van safrol door gebruik te maken van op de fysiologie gebaseerde biokinetische (PBBK) modellen.

In het eerste hoofdstuk van dit proefschrift wordt achtergrondinformatie gegeven over het onderwerp en worden de doelen van het proefschrift gepresenteerd. In hoofdstuk 2 wordt een PBPK-model ontwikkeld, dat is gebaseerd op in *in vitro* experimenten bepaalde metabole parameters, *in silico* bepaalde partitiecoëfficiënten, en fysiologische paramaterwaarden die zijn gevonden in de literatuur (Brown *et al.*, 1997). Het PBBK-model bestond uit 8 compartimenten waaronder lever, nier en long als metaboliserende compartimenten en daarnaast compartimenten voor vet, veneus en arterieel bloed, sterk doorbloed weefsel en zwak doorbloed weefsel. De uitkomsten van het model werden geëvalueerd door vergelijking van voorspelde niveaus van 1,2-dihydroxy-4-allylbenzene (DHAB), 1′-hydroxysafrol glucuronide (1′-HSG) en het totaal aan urinaire safrolmetabolieten met de beschreven niveaus van deze metabolieten in de urine van ratten die waren blootgesteld aan safrol (Benedetti *et al.*, 1977; Klungsøyr and Scheline, 1983). Deze evaluatie liet zien dat de voorspellingen goed overeenkwamen met de waargenomen experimentele waarden. Met het verkregen model werd vervolgens onderzoek gedaan naar de relatieve omvang van bioactivering en detoxificering van safrol bij verschillende orale doses. Zowel bij hoge als bij lage doses komt vooral in de lever conversie van safrol voor, gekatalyseerd door cytochroom P450 (P450) waarbij DHAB als voornaamste P450-metaboliet van safrol kan worden verwacht. Een dosis- afhankelijke verschuiving in P450-gemedieerde omzetting naar een relatieve toename in bioactivering bij hoge doses werd niet waargenomen. Dit kan worden verklaard door het feit dat voor P450- gemedieerde omzetting van safrol geen verzadiging van een belangrijke metabole route voorkomt in een dosisreeks van 0.05 tot 300 mg/kg lichaamsgewicht. Extrahepatische conversie van safrol draagt niet significant bij aan het safrolmetabolisme. In mannelijke rattenlever is glucuronidering de belangrijkste route voor de omzetting van 1′-hydroxysafrol, gevolgd door oxidering terwijl sulfatering van 1′-hydroxysafrol een minder belangrijke route is maar wel de route die de uiteindelijke carcinogene metaboliet 1′-sulfooxysafrol genereert.

In hoofdstuk 3 van dit proefschrift wordt een PBPK-model voor safrol in de mens beschreven dat is gebaseerd op via *in vitro*- en *in silico*- experimenten bepaalde parameters. Het model bestaat uit 8 compartimenten, waaronder lever, nier en long als metaboliserende compartimenten, en compartimenten voor vet, veneus en arterieel bloed, en sterk- en

minder sterk doorbloed weefsel. Met het verkregen model werden de tijd- en dosisafhankelijke vorming van de voorloper van de carcinogene metaboliet 1′-hydroxysafrol en van de carcinogene metaboliet 1′-sulfooxysafrol, in menselijke lever berekend. Aanvullend werd een Monte Carlo-simulatie uitgevoerd om interindividuele variatie te voorspellen in de vorming van deze metabolieten in de populatie als geheel. Het PBBK-model voor de mens werd geëvalueerd door de voorspelde PBBK en de beschreven experimentele data te vergelijken op het niveau van de totale safrol metabolietvorming, gemeten in de urine van menselijke vrijwilligers die waren blootgesteld aan safrol (Benedetti *et al*, 1977) wat resulteerde in een goede match. De vergelijking van het PBBK-model voor rat en mens liet zien dat het verwachte niveau van vorming van 1′-hydroxysafrol in humane lever vier maal hoger is dan dat in rattenlever en de voorspelde vorming van 1′-sulfooxysafrol is ongeveer vijf keer hoger in humane lever dan in rattenlever. Dit toont aan dat de interspeciesverschillen in toxicokinetiek voor bioactivering van safrol tussen ratten en mensen in overeenstemming zijn met de onzekerheidsfactor van 4 die normaal gesproken in acht wordt genomen voor interspeciesverschillen in toxicokinetiek (IPCS, 2010). Speciesverschillen tussen mensen en ratten wat betreft de eigenschap van de detoxificeringsroutes van 1′-hydroxysafrol waren groter. De vorming van 1′-oxosafrol is de belangrijkste detoxificeringsroute in de mens, maar een minder belangrijke route in de rat en glucuronidering van 1′-hydroxysafrol is de belangrijkste detoxificeringsroute in de rat, maar een minder belangrijke route in de mens. Het PBBK-model voorspelde dat de vorming van 1′-sulfooxysafrol in de lever van de mens en de rat een lineair verband laat zien met de dosis, over het hele gebied van dosisniveaus waarvan bekend is dat deze kanker veroorzaken in knaagdier-bioassays (100 mg/kg lichaamsgewicht) naar de dosisniveaus die relevant zijn voor de dagelijkse menselijke inname en even laag als de virtuele veilige dosis (vitual safe dose, VSD). Dit betekent dat PBBK-modellen de interspeciesverschillen kunnen evalueren en een nuttige bijdrage kunnen leveren in de risk assessment van in voedsel voorkomende chemicaliën bij het evalueren van menselijke risico's. Monte Carlo-simulaties toonden aan dat de vorming van 1′-sulfooxysafrol in de populatie 4-voudig tot 17-voudig bleek te verschillen (verschil tussen achtereenvolgens de 95^{e} percentiel en de mediaan, en 95^{e} en 5^{e} percentiel).

Bovendien kan een CSAF (chemical-specific adjustment factor) worden bepaald voor interindivuele verschillen in kinetiek voor voorspelde vorming van 1′-sulfooxysafrol, berekend als de ratio van de parameter van het 95^{e} percentiel en de mediaan, die uitkomt op 3.9 (IPCS, 2005). Vergelijking van deze CSAF met de standaard onzekerheidsfactor van 3.16 voor interspeciesvariabiliteit in biokinetiek (ervan uitgaand dat een factor 10 evenredig verdeeld is in twee sub-factoren van elk $10^{-0.5}$ (3.16) voor biokinetiek en biodynamiek) (IPCS, 2010) laat zien dat de standaard onzekerheidsfactor van 3.16, 91% van de populatie voldoende beschermt.

Om de dagelijkse inname te reduceren, heeft het huidige EU-voorschrift No. 1334/2008 toevoeging van safrol als pure stof aan voeding verboden Als gevolg van deze regels komt de huidige inname van safrol door de mens voornamelijk door het gebruik van specerijen die safrol bevatten, zoals foelie, nootmuskaat, peper etc., of door voeding waaraan deze specerijen of de essentiële oliën daarvan zijn toegevoegd. Daarom moet risk assessment van safrol door consumptie van kruiden en specerijen die safrol bevatten, worden uitgevoerd met inachtneming van het mogelijk modulerend effect van andere stoffen die in die kruiden of specerijen zitten. De gevolgen van mogelijke matrixafhankelijke effecten op de bioactivering en detoxificering van safrol zijn onderzocht in hoofdstuk 4. Omdat bekend is dat foelie relatief hoge niveaus van safrol bevat (Archer, 1988) werd foelie-extract, en fracties daarvan, getest op remming van SULT-activiteit, de reactie die verantwoordelijk is voor de laatste stap in de bioactivering van safrol. De foelie-fractie die malabaricone C bevatte, vertoonde de hoogste SULT-remmingsactiviteit. De K_i (remmingsconstante) voor remming van SULT-activiteit door het malabaricone C-bevattende extract was 8.3 en 1.0 mg/L voor respectievelijk ratten- en mensenlever S9. Remming door foelie-extract van oxidering en glucuronidering van 1′-hydroxysafrol werd niet gevonden bij een foelie-extractconcentratie van 100 mg/L en dat toont aan dat foelie-extract SULTs selectief remde. In hoofdstuk 4 bewijzen verdere studies met menselijke HepG2 cellen blootgesteld aan 1′-hydroxysafrol in aan- en afwezigheid van foelie-extract, dat de vorming van het DNA adduct *N*^{2}-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine (S-3′-*N*^{2}-dGuo) werd geremd voor 60 en 80% bij foelie-extraxtconcentraties van respectievelijk 25 en 100 mg/L. Het foelie-

extract vertoonde geen cytotoxiciteit tot concentraties van ten minste 100mg/L, de hoogst geteste concentratie.

Om te onderzoeken of deze effecten op safrol-bioactivering via remming van SULT gemedieerde omzetting van 1′-sulfooxysafrol door malabaricone C- bevattende foelie-extract eveneens *in vivo* kunnen worden verwacht, werd de SULT-remming geïntegreerd in het PBBK-model op basis van de *Ki*-waarden voor ratten- of humaan S9. Het PBBK-model dat op die manier werd verkregen, gaf aan dat bij een dosis van 50 mg/kg lichaamsgewicht safrol en een dosis van malabaricone C-bevattend foelie-extract in een ratio gelijk aan het niveau van deze bestanddelen in foelie (44 mg/µmol), remming van 1′-sulfooxysafrolvorming door malabaricone C-bevattend foelie-extract voor ratten en mensen op zal treden op een niveau van 90 en 100%, wanneer de biobeschikbaarheid van de foelie-extractingrediënten 100% zou zijn. Dit toont een potentiële reductie aan van bioactivering en van het kankerrisico als safrol-inname een gevolg is van de voedselmatrix die malabaricone C- of andere SULT-remmers bevatten in het reguliere dagelijkse dieet, vergeleken met resultaten die verkregen zijn bij dosering van safrol als pure stof.

In hoofdstuk 5 werd onderzocht of de remming van safrol DNA-adductvorming door malabaricone C-bevattend foelie-extract, waargenomen in het *in vitro* model systeem, ook gezien kan worden in een *in vivo* experiment. Daartoe werden Sprague-Dawley ratten oraal blootgesteld aan foelie-extract en safrol en het niveau van safrol DNA-adducten die in de lever werden gevormd, werd gekwantificeerd en vergeleken met het niveau van safrol DNA-adducten die gevonden zijn in de lever van ratten die alleen aan safrol zijn blootgesteld. De verkregen resultaten toonden aan dat safrol DNA-adductvorming in de lever van Sprague-Dawley ratten door het foelie-extract met 55% werd gereduceerd. Het PBBK-model voorspelde dat een 55% remming van safrol bioactivering op 1′-sulfooxysafrol zou worden verkregen als de biobeschikbaarheid van de SULT-remmers in het malabaricone C-bevattend foelie-extract 68% zou zijn in plaats van 100%, wat mogelijk niet onrealistisch is. De resultaten van de *in vivo* studie komen dus overeen met de op het PBBK-model gebaseerde voorspellingen voor safrol bioactivering. De *in vivo* studie liet ook een remming zien van 64%van de safrol DNA-adductvorming in de lever bij combinatieblootstelling van de ratten aan safrol en de bekende SULT-remmer PCP. Dit

resultaat komt overeen met eerder beschreven resultaten in ratten of muizen die werden blootgesteld aan safrol of 1′-hydroxysafrol, gecombineerd met PCP (Boberg *et al*., 1983; Daimon *et al*., 1997; Randerath *et al*., 1984). In hoofdstuk 5 is ook aangetoond dat urinaire excretie van de belangrijkste safrolmetabolieten, (geconjugeerde) hydroxysafrol en DHAB niet significant was gereduceerd als safrol gelijktijdig werd gedoseerd met foelie-extract of PCP. Deze waarneming sluit uit dat de reductie in DNA-adductvorming het gevolg is van een gereduceerde biobeschikbaarheid van safrol als die samen met malacaribone C-bevattend foelie-extract of PCP wordt gedoseerd.

Hoofdstuk 6 van het proefschrift is een algemene discussie over de verkregen resultaten. Met het PBBK-model dat is ontwikkeld voor ratten en mensen, kan de tijd- en dosis-afhankelijke vorming van de voor bioactivering relevante carcinogene metabolieten, 1′-hydroxysafrol en 1′-sulfooxysafrol, in ratten- en mensenlever worden voorspeld. Menselijke interindividuele verschillen in bioactivering van safrol kan worden gesimuleerd door Monte Carlo-simulatie te gebruiken. Bovendien zijn de voorspellingen door de PBBK-modellen voor rat en mens vergeleken om de speciesverschillen in bioactivering en detoxificering van safrol te evalueren. De resultaten van de *in vitro* en *in vivo* studies die remming vertoonden van de vorming van safrol DNA-adducten door foelie-extract, bevestigen dat in risk assessment rekening moet worden gehouden met gecombineerde effecten als safrol zou worden getest in combinatie met een relevante voedselmatrix. Om de van de voedselmatrix afhankelijke modulering van safrol bioactivering in de risk assessment van safrol te integreren, kan de zogeheten Margin of Exposure (MOE) worden gebruikt. Deze benadering laat zien dat, als safrol zou worden getest in knaagdierbioassays samen met een matrix die SULT-remmers bevat, de MOE-waarden hoger zouden zijn en de noodzaak voor risk management geringer. Tot slot geeft hoofdstuk 6 enkele onderwerpen die voor verder onderzoek naar bioactivering en risk assessment van safrol van belang zouden zijn.

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Appendix

Abbreviations

Acknowledgements

Curriculum vitae

List of publications

Overview of completed training activities

Abbreviations

1′HOS, 1′-oxosafrole

1′HS, 1′-hydroxysafrole

1′HSG, 1′-hydroxysafrole glucuronide

1′HSS, 1′-sulfooxysafrole

7HC, 7-hydroxycoumarin

7HCS, 7-hydroxycoumarin sulphate

ADME, absorption, distribution, metabolism and excretion

ALARA, as low as reasonably achievable

BMD, benchmark dose

$BMDL_{10,}$ lower confidence limit of the benchmark dose that gives 10% increase in tumor incidence over background level

bw, body weight

CEFS, Committee of Experts on Flavouring Substances

CSAF, chemical-specific adjustment factor

CV, coefficient of variation

DAD, diode array

DHAB. 1,2-dihydroxy-4-allylbenzene

DMSO, dimethyl sulfoxide

E-3′-N^2-dGuo, N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine

EDI, estimated daily intake

EFSA, European Food Safety Authority

EU, European Union

F, female

FDA, Food and Drug Administration

GRAS, generally recognized as safe

GSH, glutathione reduced

h, hour

i.p, intraperitoneal

JECFA, Joint FAO/WHO Expert Committee on Food Additives

K, kidney

K_i, inhibition constant

L, liver

LC, liquid chromatography

LC-MS, liquid chromatography–mass spectrometry

Lu, lung

M, male

MOE, margin of exposure

MPL, microsomal protein yield of the liver

MTT, 3-(4,5-dimethylthiazol-2-y1) 2, 5-diphenyl tetrazolium bromide

NAD, β-nicotinamide adenine dinucleotide

NADPH, reduced β-nicotinamide adenine dinucleotide

NOAEL, no observed adverse effect level

nt, nucleotide

P450, cytochrome P450 enzyme

PAPS, 3′-phosphoadenosine-5′-phosphosulphate

PBBK, physiologically based biokinetic

PCP, pentachlorophenol

s.c, subcutaneous

s.t, stomach tube

S-1′-N^2-dGuo, N^2-(safrol-1′-yl)-2′-deoxyguanosine

S-3′-N^2-dGuo, N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine

S-3′-N^6-dAdo, N^6-*(trans*-isosafrol-3′-yl)-2′-deoxyadenosine

S9PL, yield of S9 protein

SCE, sister chromatid exchange

SCF-EU, Scientific Committee on Food of the European Union

SCOGS, Select Committee on GRAS Substances

SD, Sprague Dawley

SPDE, spleen phosphodiesterase

SULT, sulfotransferase

UDPGA, uridine 5′-diphosphoglucuronic acid

UGT, UDP-glucuronosyltransferase

UPLC, ultra performance liquid chromatography

VPDE, venom phosphodiesterase

VSD, Virtual Safe Dose

wk, week

Acknowledgements

I would like to express my deepest gratitude to my promotor Ivonne Rietjens for continuous support of my PhD and immense knowledge. Burden of finalizing of this thesis was substantially reduced by her very excellent and very efficient supervising. My sincere thanks also go to my other promotor Peter van Bladeren for critical comments to the manuscripts and for valuable meetings during his visits to Wageningen. I would like to show my greatest appreciation to my co-promotor Ans Punt from whom I learned a lot about PBBK modeling.

I would like also to thank Bert Spenkelink for always taking a quick action to solve the problems with the HPLC and telling nice stories and details about dutch habits, Marelle Boersma for her help with HPLC, Laura de Haan for introducing me to work with cell lines, Hans van den Berg for helping me with the in vivo experiments, Ans Soffers for checking the PBBK model results, Jacques Vervoort for keeping the LC-MS/MS work running and Rene Bakker and his colleagues for excellent assistance during the animal experiment. Special thanks to Irene Keultjes and Gré Schurink for their great managing of the administration especially regarding my presence in the Netherlands.

Furthermore, I want to thank the staff, (former) PhD students and my students at Toxicology: Tinka, Gerrit, Jac, Jochem, Henrique, Jaimes, Merel, Wasma, Alicia, Suzanne, Nynke, Arif, Reiko, Rungnapa, Alex, Hequn, Marije, Myrthe, Jonathan, Justine, Karsten, Samantha, Sophie, Si, Elise, Sunday, Agata and Dambar. Thank you all for your help, nice conversations and friendship.

Special thanks as well to my roommates Walter, Linda, Barae and Rozaini for giving a nice environment at room 1021.

Many thanks and appreciations go to my best friends Devi, Rini and Wulan and to all my Indonesian friends in Wageningen, terimakasih atas dukungan dan kebersamaannya. Thanks to Pak Hady family yang sudah pernah menampung saya sementara di rumahnya yang asri. Terimakasih kepada pak Asep yang sudah membantu mengedit naskah, Jimmy untuk template dan tipsnya, dan Adi yang pernah memperbaiki laptop.

I must thank also all my colleagues from my home institution for supporting me to do a PhD abroad. Looking forward to rejoin with you all soon.

Most of all, I would like to thank my parents for their love and for always encouraging me, my brothers and sisters for giving me a homey atmosphere during my holiday.

Thank you all for support and encouragement during my nice and memorable journey!

Erryana

Curriculum vitae

Erryana Martati was born in Yogyakarta, Indonesia November 26th, 1969. She completed her primary education in Yogyakarta. After finishing undergraduate studies in Food Science and Technology at Gadjah Mada University (GMU), she worked at a national company of herbal medicines in Jakarta. In 1995, she left the company to pursue a master study in Food Science and Technology in GMU in Yogyakarta fully funded by the Directorate General of Higher Education (DGHE) Ministry of National Education. In 1999, she joined Department of Food Science and Technology Brawijaya University in Malang East Java. Her main activities as a permanent lecturer were teaching, conducting research and community services. In 2006, she was in a team to prepare a 3-year project proposal of Indonesia Managing Higher Education for Relevance and Efficiency (IMHERE) with the topic of Strengthening University Social Responsibility to Enhance Human Quality Relating to Sustainable Local Resources and Food Safety. In 2007, she was granted a fellowship from the Netherlands Fellowships Programme (NFP/TP) for a short course of governing food safety in international trade at Wageningen International. In 2007, she was appointed as the head of Department of Food Science and Technology Brawijaya University. In 2009, she resigned from this position and starting doing her PhD at the Toxicology Division at Wageningen University based on a scholarship from DGHE Ministry of National Education. After her PhD, she will be back to Brawijaya University to continue her academic career.

List of Publications and Abstract

Publications

Martati, E., Boersma, M. G., Spenkelink, A., Khadka, D. B., Punt, A., Vervoort, J., van Bladeren, P. J. and Rietjens, I. M. C. M (2011). Physiologically based biokinetic (PBBK) model for safrole bioactivation and detoxification in rats. *Chemical Research in Toxicology* ***24****(6): 818-834.*

Martati, E., Boersma, M. G., Spenkelink, A., Khadka, D. B., van Bladeren, P. J., Rietjens, I. M. C. M. and Punt, A (2012). Physiologically based biokinetic (PBBK) modeling of safrole bioactivation and detoxification in humans as compared With Rats. *Toxicological Sciences* ***128****(2): 301-316.*

Martati, E., Punt, A., Boersma, M. G., Spenkelink, A., Boonpawa, R., Paini, A., van Bladeren, P. J., Vervoort, J. and Rietjens, I. M. C. M. Identification of malabaricone C as an important spice constituent inhibiting safrole bioactivation and physiology-based biokinetic modeling of its possible in vivo effect. *Submitted for publication to Toxicology Letter.*

Martati, E., van den Berg, H., Vervoort, J. and Rietjens, I. M. C. M. Malabaricone C-containing mace extract inhibits safrole DNA adduct formation in the liver of orally exposed rats. *In preparation.*

Abstracts

Martati, E. and Rietjens, I.M.C.M. Physiologically based kinetic modeling and matrix mediated modulation of the bioactivation and detoxification of the allylalkoxybenzene safrole. Oral presentation at Indonesian Student Association. Wageningen. March 2010.

Martati, E., Boersma, M.G., Spenkelink, A., Khadka, D.B., Punt,A., Vervoort, J., van Bladeren, P. J and Rietjens, I.M.C.M. A Physiologically based kinetic (PBK) model for safrole bioactivation and detoxification in rat and comparison to those for estragole and methyleugenol. Poster presentation at Annual Meeting of Dutch Society of Toxicology (NVT, Nederlandse Vereniging voor Toxicologie), 17-18 Mei 2011, Zeist, The Netherlands.

Martati, E., Boersma, M.G., Spenkelink,A., Khadka, D.B., Punt,A., Vervoort, J., van Bladeren, P. J and Rietjens, I.M.C.M. A Physiologically based kinetic (PBK) model for safrole bioactivation and detoxification in rat and comparison to those for estragole and methyleugenol. Poster presentation at Annual Meeting of Association of European Toxicologists and Societies of Toxicology (EUROTOX), 28-31 August, 2011, Paris, France.

Martati, E., Boersma, M.G., Spenkelink, A., Khadka, D.B., van Bladeren, P.J., Rietjens, I.M.C.M and Punt, A. Physiologically based biokinetic (PBBK) modeling of safrole in humans as compared with rats.. Poster presentation at Annual Meeting of Association of European Toxicologists and Societies of Toxicology (EUROTOX), June 17-20, 2012, Stockholm, Sweden.

Overview of completed training activities

Discipline-specific courses

Organ Toxicology (Postgraduate Education in Toxicology (PET), 2009)
PBPK Modeling (Toxicology Dept. WUR, 2009)
Molecular Toxicology (PET, 2010)
Toxicogenomics (PET, 2010)
Advanced Food Analysis (VLAG, 2010)
Mutagenesis and Carcinogenesis (PET, 2011)
Course on Laboratory Animal Science (art.9) (Utrecht Univ., 2011)
Cell Toxicology (PET, 2011)

General courses

PhD Week (VLAG, 2009)
Information Literacy (WGS, 2010)
Health Food Innovation: Research Development and Claim Substantiation (MU, 2010)
Mini symposium "How to write a world class paper" (WUR, 2011)
Adobe in Design (WUR Library, 2011)
Scientific Writing and Presentation Skills (WGS, 2011)
Risk Assessment (PET, 2011)

Optional activities

Preparation research proposal (2009)
Research and literature discussions at the Division of Toxicology WUR (2009-2012)
Attending scientific presentation at the Indonesian Student Association (2009-2011)

Attended conferences

Annual Conferences of the 'Nederlandse Vereniging voor Toxicologie' (NVT) 2011 in Zeist, The Netherlands (poster presentation)
International Conference EUROTOX 2011 in Paris, France (poster presentation)
International Conference EUROTOX 2012 in Stockholm, Sweden (poster presentation)

Approved by graduate school VLAG

The studies described in this thesis was financially suppported by the Directorate General of Indonesia Higher Education Ministry of National Education Republic of Indonesia

Financial support from Wageningen University and Brawijaya University for printing this thesis is gratefully acknowledged

Cover photograph: Ronny Adolof Buol from Sitaro North Sulawesi

Cover design: William Setiawan

Printing: GVO drukkers en vormgevers B.V/Ponsen&Looijen, Ede, The Netherlands